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EXPERIMENTAL INOCULATION OF HUMAN THROATS WITH VIRULENT DIPHTHERIA BACILLI

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In previous reports * we have called attention to certain points concerning healthy carriers of diphtheria bacilli.

1. Such carriers are relatively abundant in the community, as may be readily demonstrated by taking throat cultures from a large series of individuals, particularly during the winter months. The number of carriers revealed by a single examination may be approximately doubled by a second examination of the same series of persons, and a third examination still further increases the number found.

2. The great majority (80 to 90 per cent) of these healthy carriers harbor diphtheria bacilli which are non-virulent. These non-virulent diphtheria bacilli are to be distinguished from the virulent ones only by their inability to produce toxin. So far as we know, this differentiation may be accomplished only by the inoculation of susceptible animals.

3. Among all of the healthy carriers of diphtheria bacilli, virulent or non-virulent, studied by us, none developed clinical diphtheria during the period of our observation, although this was in many cases extended over several months and even years.

4. A painstaking investigation failed to reveal a single case of clinical diphtheria developing among any of the associates of the healthy carriers under our observation.

5. By inoculating the throats of a series of five human volunteers with non-virulent diphtheria bacilli obtained from the throat of a healthy carrier it was shown that:

(a) The carrier state was readily produced in all, lasting from 11 weeks to 15 months or more.

(b) The development of the carrier state was not prevented by the previous injection of antitoxin in certain members of the group.

(c) Confirmation of the guinea-pig test for the avirulence of this organism was obtained by these experiments, in that this organism which was shown by repeated tests to be avirulent for guinea-pigs failed to produce (a) clinical diphtheria; (b) any subjective symptoms; (c) any objective changes in

* Moss, W. L., Guthrie, C. G., and Gelien, J.: Diphtheria Bacillus Carriers, Trans. XV Internat. Cong. on Hyg. and Dem., 1912, IV, 156.

Guthrie, C. G., Gelien, J., and Moss, W. L.: Diphtheria Bacillus Carriers, Second Communication. Johns Hopkins Hosp. Bull., 1920, XXXI, 388.

Moss, W. L., Guthrie, C. G., and Marshall, B. C.: Experimental Inoculation of Human Throats with Avirulent Diphtheria Bacilli. Johns Hopkins Hosp. Bull., 1921, XXXII, 37.

Moss, W. L., Guthrie, C. G., and Gelien, J.: Diphtheria Bacillus Carriers. A Report of Conditions Found in an Orphan Asylum. Johns Hopkins Hosp. Bull., 1921, XXXII, 109.

the appearance of the throat when inoculated and colonized in human throats.

(d) No cases of clinical diphtheria developed among the associates of these artificially produced "healthy carriers" of avirulent diphtheria bacilli.

(e) When isolated in pure culture after prolonged sojourn in the throats of these carriers, the bacilli were found unaltered in morphology, in staining or cultural characteristics, and showed no tendency to become virulent, either in the carriers who had received preliminary injections of antitoxin or in those who had not.

6. The results of the foregoing observations on individuals in the carrier state, whether spontaneously or experimentally produced, warranted the general conclusions that non-virulent diphtheria bacilli are incapable of producing clinical diphtheria and that carriers of such organisms are not a factor in the spread of the disease.

These conclusions still leave for consideration, however, the healthy carriers of virulent diphtheria bacilli regarding whom certain questions naturally arise. With the exception of diphtheria bacilli isolated from actual cases of clinical diphtheria we have in the past relied on the guinea-pig test for determining the virulence or avirulence of a given strain. The question has often been raised as to whether the demonstration of virulence for the guinea-pig may be taken as proof of virulence for man. While the validity of the guinea-pig test as indicating virulence for man is generally accepted and there is much evidence in its favor, the conclusive proof, so far as we are aware, had never been brought and certain facts seemed to leave the question an open one. These facts may be indicated by stating the questions which arose concerning healthy carriers of virulent diphtheria bacilli. The first question concerns the reason why such carriers do not themselves develop clinical diphtheria. Is it because they are protected against the effect of diphtheria toxin by means of an immunity either naturally present prior to infestation or gradually acquired as the result of the presence of virulent bacilli in the throat? Or is it because the virulent organism from the throat of a healthy carrier, though virulent for the guinea-pig, differs in some way from the virulent organism from a patient with diphtheria and so is incapable of producing the disease in human beings? In other words, is the guinea-pig test misleading in this respect? The second question concerns the reason why no discoverable case of clinical diphtheria occurred among the associates of the healthy carriers of virulent diphtheria bacilli. This question again brings up the validity of the guinea-pig test for virulence. Do such carriers fail to transmit the disease to others because the organisms which they harbor, though virulent for the guinea-pig, are incapable of producing diphtheria in man? It was in an endeavor to find the answer to these and certain other questions that the present work was undertaken. It is largely a repetition of the series of experiments carried out in the previous year (1913), in the course of which we inoculated the throats of human beings with non-virulent diphtheria bacilli. The only essential difference lies in the strain of organism used for our inoculations, which, in the work to be presented here, was a virulent diphtheria bacillus obtained from the throat of a healthy carrier. Since it parallels the previous work so closely, the form of the earlier report is also followed here; in many places the identical wording is retained.

Object

1. To determine whether the introduction of virulent diphtheria bacilli (as determined by guinea-pig inoculation) from the throat of a healthy carrier into the throats of healthy human beings free from diphtheria bacilli will result in the production of the carrier state and, if produced, how long it may last.

2. To determine whether the lodgment and growth of the bacilli may be prevented by the previous injection of diphtheria antitoxin.

3. To determine whether the organisms introduced are capable of producing (a) clinical diphtheria; (b) any subjective symptoms; (c) any objective signs in the appearance of the throat.

4. To determine whether any cases of clinical diphtheria develop among the associates of artificially produced "healthy" carriers of such virulent diphtheria bacilli obtained from the throat of a healthy carrier.

5. To determine whether the organisms introduced into the throats of normal human beings are in any way changed by this procedure, particularly as to (a) morphology; (b) staining characteristics; (c) cultural characteristics; (d) ability to produce toxin (virulence).

Procedure

Eight persons working in the laboratory (W. L. M., C. G. G., A. W. S., D. C. S., B. C. M., S. E. S., F. E. and W. H.) volunteered to be the subjects of these experiments. These individuals—designated for the sake of brevity in the following pages as A, B, D, V, W, X, Y and Z—were all healthy adults, varying in age from 23 to 37 years.* Three of them (A, B and D) were members of the group used in our experiments of the year before (1913), while the others (V, W, X, Y and Z) were new material. It was our original intention to include the two other persons, C and E (S. R. M. and W. A. B.), who had volunteered for the earlier series of experiments, but they were still artificially-produced carriers of non-virulent diphtheria bacilli. These two men were cultured almost daily from January 12 to May 29, 1914, but continued to be carriers throughout and so were not available for the present work. Of the eight persons who were the subjects of these experiments, two had had clinical diphtheria; D had had a severe attack at the age of five, before the introduction of antitoxin; W had had an attack at the age of six; both had recovered without the use of antitoxin. Only one had previously received antitoxin; D had received 250 units in February, 1913, almost a year before the present work was begun, as a part of the

* Five members of this group were physicians, two were medical students and one was a laboratory assistant conversant with experimental methods and especially familiar with diphtheria.

experiments undertaken at that time. None of these individuals has contracted diphtheria or received antitoxin since the close of the experiments reported here, but four of them (B, V, W and X) developed diphtheria for which they received curative doses of antitoxin in the course of this work, as will be mentioned later. Schick tests performed on these individuals showed that four (B, V, W and X) were without circulating antitoxin, as indicated by a strongly positive reaction at each trial. The tests on Y and Z were always negative, indicating some degree of immunity against diphtheria toxin. A and D showed "pseudo-positive" reactions with unheated toxin, as indicated by the occurrence of a reaction of similar character and intensity when heated toxin was used; these reactions are to be interpreted as negative and A and D are, consequently, to be grouped with Y and Z as being relatively immune against diphtheria toxin.

Preliminary throat cultures were begun on the five persons (A, B, C, D and E) used in our earlier experimental inoculations with non-virulent bacilli, on January 12, 1914. It was soon found, as was mentioned above, that C and E were still carriers of non-virulent organisms and so unavailable for the purposes of the present work. After five successive negative cultures, D showed one positive culture on January 17, containing organisms of the type with which he had been inoculated 11 months before. For this reason it was feared that he also could not be used for these experiments but the daily preliminary cultures were continued for a long period—until April 2 in the case of all members of the group except A and B—and no further positives were encountered. Additional persons were added to the group from time to time (V on January 13, W on January 18, X on January 19, Y on January 20 and Z on March 9), and preliminary cultures were continued on the entire group to ascertain whether any of the members had diphtheria bacilli in their throats at the beginning of the experiments.

For the purposes of the investigation it was planned to protect two members of the group and leave the other six without artificial protection. Accordingly Y and Z were each given a subcutaneous injection of 1000 units of diphtheria antitoxin, 24 hours prior to inoculation of their throats with virulent diphtheria bacilli.

The strain of diphtheria bacillus employed in this work (Culture 198) was obtained by us from a healthy carrier during an investigation of public school children in 1912 and, at the time of the experiments reported in the present paper (1914), it had been growing on Loeffler's blood serum for over 24 months.* When first isolated the strain proved to be virulent; repeated tests over a period of two years—20 or more, including those made at the beginning of this work—in each instance confirmed the original result. Morphologically, tinctorially and culturally the strain was typical *B. diphtheriæ*, indistinguishable from our other virulent strains and distinguishable from our non-virulent strains only by the animal test for the production of toxin.

The inoculations were made from transplants of this strain grown on Loeffler's blood serum for 24 hours and then suspended in normal salt solution. By using the growth from several tubes, this saline suspension was rendered quite thick and opaque.

The inoculations were made by soaking a cotton swab in the bacterial suspension and then rubbing it over both tonsils and the posterior pharyngeal wall. As a result of this procedure, all members of the group except Y soon showed positive cultures, so that further inoculations were considered unnecessary; by the time it became evident that Y had not developed the carrier state, our earlier enthusiasm had cooled considerably and she was not subjected to reinoculation.

As the inoculation with virulent diphtheria bacilli was believed to be a somewhat more hazardous undertaking than that carried out with non-virulent organisms in the preceding year, it was decided to try the effect of the experiment first on A and B (W. L. M. and C. G. G.). Accordingly, these two members of the group were inoculated on March 18 in the manner indicated above. Although the results of the inoculation of these two persons were known to all of the group, the other members were willing to proceed with the experiment and they were inoculated also, D, V, W, Y and Z on April 2, and X on April 3.

Daily cultures were made from the various persons engaged in the experiment from the date of inoculation until May 29 of the same year.

RESULTS

The results of the cultures taken from January 12 to May 29, 1914, are shown in the accompanying chronological table (Table I). In this table a *positive* culture is indicated by +; a *negative* culture by 0; a day on which no culture was taken is indicated by ..; † indicates a subcutaneous injection of diphtheria antitoxin;* indicates inoculation of the throat with virulent diphtheria bacilli.

From Table I it will be seen that in all of the preliminary cultures taken prior to inoculation only one positive culture was found, that obtained from D on January 17. This culture contained non-virulent but otherwise typical diphtheria bacilli, indistinguishable from those with which his throat had been inoculated 11 months before and which were responsible for the production of the carrier state. Frequent cultures were made from D from the date of this positive culture until April 2—55, in all over a period of 75 days—and no further positives were encountered. We felt justified, therefore, in assuming that none of the eight individuals who had volunteered for these experiments were carriers of diphtheria bacilli at the time of inoculation.

On March 18, two members of the group (A and B) were inoculated by swabbing the throats with a saline suspension of virulent diphtheria bacilli obtained from the throat of a

* Concerning the child from whose throat this strain (Culture 198) was obtained, no history of diphtheria or of exposure to diphtheria previous to our investigation could be obtained; during the period covered by our observations which extended over several months, the child did not develop diphtheria nor did any case of diphtheria occur among the child's most intimate associates.

healthy carrier, as described above. The results of this procedure were strikingly different in these two individuals.

From A, who had a negative Schick test and therefore presumably had circulating antitoxin in his blood, throat cultures were negative for five days following inoculation. On the sixth day, however, his culture was positive and again on the seventeenth, the twentieth, and thirty-third day following inoculation, with 12 negative cultures between the first and last positive. After this he had 22 negative cultures in the 39 days to May 29, the end of our period of observation.

TABLE I

Day	Date	A	B	D	V	W	X	Y	Z	Day	Date	A	B	D	V	W	X	Y	Z	Day	Date	A	B	D	V	W	X	Y	Z
1	Jan. 12	0	0	0	..	..	..	..	..	47	Feb. 27	0	0	0	0	0	..	0	..	93	Apr. 14	..	..	+	+	..	+	..	+
2	Jan. 13	0	0	0	0	..	..	..	..	48	Feb. 28	0	0	0	0	0	..	0	..	94	Apr. 15	..	0	..	+	+	+	0	0
3	Jan. 14	..	0	0	0	..	..	..	..	49	Mar. 1	0	0	0	..	0	..	..	..	95	Apr. 16	..	0	..	+	+	+	0	0
4	Jan. 15	0	0	0	0	..	..	..	..	50	Mar. 2	0	0	0	0	0	..	0	..	96	Apr. 17	0	+	+	+	+	+	0	+
5	Jan. 16	0	0	0	0	..	..	..	..	51	Mar. 3	0	0	0	0	0	0	0	..	97	Apr. 18	..	..	..	+	+	0	..	0
6	Jan. 17	0	0	+	0	..	..	..	..	52	Mar. 4	0	0	0	0	0	0	0	..	98	Apr. 19	..	0	..	..	+	+	0	+
7	Jan. 18	0	0	..	0	0	..	..	..	53	Mar. 5	0	0	..	0	0	..	0	..	99	Apr. 20	+	..	..	..	+	+	0	0
8	Jan. 19	0	0	0	0	0	0	..	..	54	Mar. 6	0	..	0	0	0	0	0	..	100	Apr. 21	0	..	..	+	+	+	0	+
9	Jan. 20	0	0	0	0	0	0	0	..	55	Mar. 7	0	..	0	..	..	..	0	..	101	Apr. 22	0	0	+	+	+	+	0	+
10	Jan. 21	0	0	0	0	0	..	0	..	56	Mar. 8	0	0	0	..	0	..	0	..	102	Apr. 23	0	0	..	..	+	+	0	0
11	Jan. 22	0	0	0	0	0	0	0	..	57	Mar. 9	0	0	0	0	0	..	0	0	1 3	Apr. 24	0	..	..	+	+	+	0	+
12	Jan. 23	0	0	0	0	0	0	0	..	58	Mar. 10	0	0	..	0	..	..	0	..	104	Apr. 25	..	0	+	+	+	+	0	..
13	Jan. 24	..	0	0	0	0	0	0	..	59	Mar. 11	0	0	0	..	0	..	0	0	105	Apr. 26	0	0	+	+	..	..	0	+
14	Jan. 25	0	0	0	0	0	0	..	..	60	Mar. 12	0	0	..	0	0	..	0	0	106	Apr. 27	..	..	..	..	..	..	..	0
15	Jan. 26	..	0	0	..	0	0	..	..	61	Mar. 13	0	0	..	0	0	..	0	0	107	Apr. 28	0	0	..	..	+	+	0	+
16	Jan. 27	0	0	..	0	0	0	0	..	62	Mar. 14	..	0	0	0	..	..	..	..	108	Apr. 29	..	0	+	..	+	..	..	0
17	Jan. 28	0	0	0	0	..	0	0	..	63	Mar. 15	..	0	0	0	0	..	..	..	109	Apr. 30	0	0	+	+	0	+	0	+
18	Jan. 29	0	0	0	..	0	0	0	..	64	Mar. 16	0	0	..	0	..	..	0	0	110	May 1	0	0	0	+	+	+	0	..
19	Jan. 30	0	0	0	0	0	0	0	..	65	Mar. 17	..	0	..	0	..	..	..	0	111	May 2	..	+	+	+	+	+	..	..
20	Jan. 31	0	0	0	..	0	0	0	..	66	Mar. 18	*	*	..	..	..	..	0	0	112	May 3	0	..	..	..	..	+	0	..
21	Feb. 1	0	0	..	..	0	..	..	..	67	Mar. 19	0	+	0	0	0	..	0	0	113	May 4	0	+	+	+	..	0	0	+
22	Feb. 2	0	0	0	0	0	0	0	..	68	Mar. 20	0	+	0	0	0	..	0	0	114	May 5	..	0	+	0.	+	+	..	..
23	Feb. 3	0	0	0	0	0	0	0	..	69	Mar. 21	0	+	0	..	0	..	0	0	115	May 6	0	..	..	+	+	+	..	..
24	Feb. 4	0	0	0	0	0	0	0	..	70	Mar. 22	0	..	0	..	0	..	0	..	116	May 7	..	+	..	+	+	+	..	+
25	Feb. 5	0	0	..	..	0	..	0	..	71	Mar. 23	0	..	0	0	0	0	0	0	117	May 8	..	..	0	+	+	+	0	0
26	Feb. 6	0	0	0	0	0	0	0	..	72	Mar. 24	+	0	0	0	0	..	0	0	118	May 9	..	..	0	+	+	+	0	+
27	Feb. 7	..	0	..	0	0	0	..	..	73	Mar. 25	0	+	0	..	..	0	0	0	119	May 10	0	0	0	+	+	..	0	..
28	Feb. 8	0	0	..	0	0	..	..	..	74	Mar. 26	..	+	..	..	0	0	0	..	120	May 11	..	0	+	+	+	..	0	+
29	Feb. 9	0	0	0	0	0	0	0	..	75	Mar. 27	0	0	0	0	0	0	0	0	121	May 12	..	0	..	+	..	+	..	0
30	Feb. 10	0	0	0	0	0	0	0	..	76	Mar. 28	0	0	0	0	0	0	0	0	122	May 13	..	0	+	+	+	+	0	0
31	Feb. 11	..	0	0	0	0	..	0	..	77	Mar. 29	..	0	0	..	..	0	0	..	123	May 14	0	+	+	+	+	+	..	..
32	Feb. 12	0	0	0	0	0	0	0	..	78	Mar. 30	..	+	..	..	0	0	0	..	124	May 15	0	+	+	+	+	..	+	0
33	Feb. 13	0	0	0	..	0	0	0	..	79	Mar. 31	..	+	0	0	0	0	0	0	125	May 16	0	0	+	+	+	+	0	+
34	Feb. 14	0	..	0	..	0	0	0	..	80	Apr. 1	0	+	..	0	..	0	0†	†	126	May 17	0	..	..	..	..	+	0	..
35	Feb. 15	..	..	..	..	0	0	..	..	81	Apr. 2	0	+	0*	*	0*	0	0*	*	127	May 18	0	0	..	..	+	+	..	0
36	Feb. 16	..	..	..	..	..	..	..	..	82	Apr. 3	0	+	+	+	0	0*	0	0	128	May 19	0	+	0	+	+	+	0	0
37	Feb. 17	0	0	0	0	0	..	0	..	83	Apr. 4	+	+	+	+	0	+	0	0	129	May 20	..	0	..	+	..	+	0	+
38	Feb. 18	0	..	0	0	0	0	0	..	84	Apr. 5	0	+	+	+	+	+	0	+	130	May 21	0	0	..	..	+	+	0	+
39	Feb. 19	0	0	0	0	0	..	0	..	85	Apr. 6	..	..	..	..	+	..	0	0	131	May 22	..	0	+	..	+	0	..	0
40	Feb. 20	0	0	0	0	0	..	0	..	86	Apr. 7	+	0	+	..	..	..	0	0	132	May 23	..	0	..	..	+	..	0	..
41	Feb. 21	0	0	0	0	0	0	0	..	87	Apr. 8	..	+	..	..	+	..	0	0	133	May 24	..	..	..	..	..	..	..	..
42	Feb. 22	..	0	..	..	0	..	..	..	88	Apr. 9	0	+	..	+	+	+	0	0	134	May 25	0	0	..	+	+	0	0	+
43	Feb. 23	..	..	..	..	..	..	..	..	89	Apr. 10	0	+	..	..	+	0	0	+	135	May 26	0	+	..	+	+	..	..	..
44	Feb. 24	0	0	0	0	0	..	0	..	90	Apr. 11	0	0	..	+	+	+	0	+	136	May 27	..	0	..	..	+	0	..	+
45	Feb. 25	0	0	0	0	0	..	0	..	91	Apr. 12	0	+	..	..	..	+	..	..	137	May 28	..	+	..	+	..	..	..	..
46	Feb. 26	0	0	0	0	0	0	0	..	92	Apr. 13	..	+	+	+	+	+	0	+	138	May 29	0	+	..	+	0	0	..	..

B, who at every test showed a strongly positive Schick reaction and therefore was presumably without natural immunity against diphtheria toxin, developed a frank attack of clinical diphtheria, following which he continued to harbor diphtheria bacilli and was still a carrier at the time of our last examination on May 29. From the date of inoculation he had 27 positive and 30 negative cultures over a period of 72 days.

On April 1, two members of the group (Y and Z) received 1000 units of diphtheria antitoxin subcutaneously. On the following day these two, together with three other members (D, V and W), who received no antitoxin, were inoculated by swabbing the throats with a saline suspension of virulent diphtheria bacilli, as described above. X, the remaining member of the group who also had received no preliminary injection of antitoxin, was inoculated in like manner on the following day, April 3. Here again the results of inoculation differed markedly in the various individuals.

D, who had received no antitoxin but who had a negative Schick test, did not develop clinical diphtheria but promptly became a carrier and remained a carrier to the last observation on May 22. During this time (50 days) there were obtained from him 21 positive and 5 negative cultures.

V, W and X who had received no antitoxin but each of whom had a positive Schick test, all developed diphtheria, following which they all became carriers and continued to habor diphtheria bacilli practically to the end of the period of observation on May 29. Following inoculation, V had 37 positive cultures and 1 negative culture; W had 41 positive and 4 negative; X had 36 positive and 7 negative.

Y and Z, each of whom had received a preliminary injection of antitoxin and who already had some degree of natural antitoxic immunity as manifested by a negative Schick test, did not develop diphtheria. The only evidence, and this must be regarded as inconclusive, that Y became a carrier, was the one positive culture which occurred 43 days after inoculation and 15 days before the last observation on May 29. Following inoculation she showed, in all, 1 positive culture and 39 negative cultures in the 57 days which elapsed to the date of her last culture on May 29. Z showed his first positive culture on the third day after inoculation. This was followed by 4 negative cultures, then came 4 positives, after which his cultures were irregularly positive and negative. He was still a carrier at our last observation on May 27. Following inoculation he showed in all 22 positive and 20 negative cultures.

It is seen, therefore, that as a result of experimental inoculation with virulent diphtheria bacilli originally obtained from the throat of a healthy carrier, the carrier state was established in seven out of eight members of the group; in one who had received a preliminary injection of antitoxin (Z) and in six who had not (A, B, D, V, W and X); in four who gave a positive reaction to the Schick test (B, V, W and X), and in three whose Schick test was negative (A, D and Z). The remaining member of the group (Y) failed to develop the carrier state and showed only one, isolated, positive culture 43 days after inoculation. This person had a negative Schick test and had also received a preliminary injection of antitoxin.

The results obtained in the course of our observations (Table I) are summarized in Tables II and III.

TABLE II

Date of Event	A	B	D	V	W	X	Y	Z
First preliminary culture	Jan. 12	Jan. 12	Jan. 12	Jan. 13	Jan. 18	Jan. 19	Jan. 20	Mar. 9
Last preliminary culture	Mar. 16	Mar. 17	Apr. 2	Apr. 1	Apr. 2	Apr. 3	Apr. 2	Mar. 31
Preliminary injection of antitoxin	.....	.....	.....	.....	.....	.....	Apr. 1	Apr. 1
Inoculation with *B. diphtheriæ*	Mar. 18	Mar. 18	Apr. 2	Apr. 2	Apr. 2	Apr. 3	Apr. 2	Apr. 2
First curative injection of antitoxin	.....	Mar. 20	.....	Apr. 5	Apr. 6	Apr. 5	.....	.....
First positive culture	Mar. 24	Mar. 19	Apr. 3	Apr. 3	Apr. 5	Apr. 4	May 15	Apr. 5
Last positive culture	Apr. 20	May 29	May 22	May 29	May 27	May 21	May 15	May 27
Last culture taken	May 29	May 29	May 22	May 29	May 29	May 29	May 29	May 27

TABLE III

Cultures	A	B	D	V	W	X	Y	Z
Total	96	115	87	95	108	83	100	58
Preliminary	53	58	61	57	63	40	60	16
After inoculation with *B. diphtheriæ*	43	57	26	38	45	43	40	42
After inoculation before first positive	5	0	0	0	2	0	31	2
Total positive	4	27	21	37	41	36	1	22
Total negative	39	30	5	1	4	7	39	20
Per cent positive	9.30	47.36	80.76	97.36	91.11	83.72	2.50	52.38

From Tables I, II and III it is seen that even the approximate time of persistence of diphtheria bacilli in the throats of these eight persons can be judged only in regard to two members of the group, A and Y. A showed only 4 positive cultures, the first 6 days and the last 33 days after inoculation. As the last positive was followed by 22 negative cultures over a period of 39 days, we may assume that A actually harbored diphtheria bacilli for at least 28 days and very likely for 33 days, since the organisms were probably present from the date of inoculation although not recovered until 6 days later. Inasmuch as Y showed only one positive culture which occurred 43 days after inoculation and this was followed by 8 negative cultures in the remaining fortnight, there is no evidence other than inferential to show that she harbored diphtheria organisms longer than one day.

Concerning B, D, V and Z, from each of whom a positive culture was obtained at the last observation, it is evident that they were carriers at least until that date and may readily have continued to be carriers for a long time thereafter. The known period during which they harbored diphtheria bacilli was, therefore, in the case of B, 72 days; for D, 50 days; for V, 57 days; and for Z, 53 days.

Neither W nor X showed a positive culture at the last examination, so we cannot be sure that either continued to harbor diphtheria bacilli after that date, but it is extremely probable that at least one and possibly both of them were carriers for a longer time than our results actually indicate. From Table I it is seen that although the last culture from W was negative, the 21 which preceded it were positive and that, of 43 cultures taken after diphtheria bacilli were first recovered from this member of the group, only 2 cultures were negative. Our evidence shows that diphtheria bacilli were present in the throat of W for at least 53 days and in that of X for at least 48 days.

It is obvious that the previous administration of 1000 units of diphtheria antitoxin did not prevent the lodgment and growth of the virulent diphtheria bacilli in the throat of Z. We cannot be perfectly sure that it did not prevent their colonization in the throat of Y; it should be pointed out, however, that although Y had only one positive culture, A, who received no preliminary injection of antitoxin, had only 4 positive cultures, so the preventive effect, if any, is not striking.

Likewise, the presence of natural immunity against diphtheria toxin did not prevent the development of the carrier state in A, D and Z, although Y, who also possessed natural antitoxic immunity as evidenced by a negative Schick test, showed only one positive culture.

The value of antitoxic immunity, natural or artificial, becomes quite apparent, however, when we come to consider the effect of the inoculation with virulent organisms obtained from the throat of a healthy carrier upon the health of these eight individuals. All of those with positive Schick tests (B, V, W and X) developed diphtheria; none of those with negative Schick tests (A, D, Y and Z) contracted the disease. Y and Z each had their natural immunity reinforced by a preliminary injection of antitoxin; so that they were spectators of, rather than participants in, whatever excitement was attendant upon the experiments.

The diphtheria from which the four less fortunate members of the group (B, V, W and X) suffered was very real, very definite, but its extent in V, W and X was wholly unpremeditated. Following inoculation of the throats of A and B on March 18, A suffered no ill effects whatever and his cultures did not become positive for 6 days, but events developed rapidly in the case of B. On the day following inoculation his culture was positive, that afternoon his throat became very sore, so much so that by suppertime he could scarcely swallow and talking became difficult. Before bedtime he had several severe chills and by the next day there was a definite diphtheritic membrane on both tonsils and extending over the soft palate toward the uvula. His temperature meanwhile rose to 103° F. and he suffered from severe headache and backache. That no question might arise as to the objective appearance of the throat, a laryngologist was consulted, who promptly made a diagnosis of diphtheria before any of the details concerning the experiment were communicated to him. B was then given a subcutaneous injection of 5000 units of diphtheria antitoxin, followed by the injection of a similar amount on the succeeding day. The membrane rapidly disappeared and the disease cleared up promptly without complications or sequelæ that were noted.

This experience served as a sharp warning of what might be expected on inoculation of susceptible individuals with virulent diphtheria bacilli from the throat of a healthy carrier, and for a time markedly diminished our zeal for further experimentation of this sort. After the lapse of a fortnight, however, our enthusiasm was partially regained and it was decided to carry out the experiment with the other members of the group. Each of them knew the result of the previous inoculations and the risks were freely discussed, but all were willing to proceed with the experiment. Accordingly, Y and Z each received a subcutaneous injection of 1000 units of antitoxin on April 1, and the following day these two, together with D, V and W, were inoculated in the same manner as that used for A and B. X was inoculated a day later. All received instructions to report at the first appearance of symptoms, as it was desired merely to test their susceptibility and to avert frank attacks of diphtheria by prompt administration of antitoxin. The results have been referred to above.

Y and Z, each of whom had a negative Schick test and had also received a preliminary injection of antitoxin, showed no ill effects following inoculation. One became a carrier, the other did not.

D, who received no preliminary injection of antitoxin but who had a negative Schick test, became a carrier, but presented no other subjective or objective effect of inoculation.

Notwithstanding the explicit instructions given, V, W and X did not report at the earliest appearance of symptoms, but only when these had become outspoken. Each of the three had a definite attack of clinical diphtheria with false membrane formation. The severity of the disease differed some-

what, W having the least and X the most severe reaction, while the intensity of the attack in V was between these two extremes. The manifestations in all three followed the same course as in B, the incubation period being practically identical, the constitutional symptoms similar and the fever outspoken in all, W reaching a maximum of 102° F., V of 102.6° F. and X of 104.7° F. Each recovered promptly following the administration of antitoxin and all became carriers.

No cases of clinical diphtheria developed among the associates of the eight members of this group.

To determine whether any change had taken place in the bacilli as the result of their residence in the human throat, the organisms were isolated at intervals from the positive cultures obtained from the various members of the group and tested culturally, tinctorially and by guinea-pig inoculation. Morphologically, the organisms were unchanged and their staining characteristics were the same as before they were introduced into the throat. On cultural tests, also, no difference was observed in the character of the growth on Loeffler's blood serum, plain agar or in bouillon, while the changes in litmus milk and in the test for fermentation of the various sugars were the same as with the original organisms. Many of these pure cultures were tested for virulence. In every instance the result was the same as with the original strain used for inoculation. There was, therefore, no evidence of loss of virulence in the bacilli as the result of their residence in the human throat.

The question of the identity of the organisms recovered from the positive cultures with the strain used for inoculation needs little discussion. The eight individuals in this group were shown to be free from diphtheria bacilli by a long series of preliminary cultures; they were then inoculated and all but one became carriers, either healthy or convalescent. The organisms which they harbored agreed with the original strain in morphology, cultural reactions and virulence.

SUMMARY

1. *Will the introduction into the throats of healthy human beings of virulent diphtheria bacilli from the throat of a healthy carrier result in the production of the carrier state and, if produced, how long may it last?*

The carrier state was produced in seven out of eight individuals by a single inoculation of the throat with virulent diphtheria bacilli obtained from the throat of a healthy carrier. The remaining member of the group showed only one positive culture which occurred 43 days after the date of inoculation. Four of these seven individuals developed clinical diphtheria and, since they continued to harbor organisms after recovery from the disease, are to be considered as convalescent carriers. The other three carriers did not contract clinical diphtheria and so may be termed healthy carriers.

The actual duration of the carrier state in any of the seven persons was not determined. It was at least 28 days in the case of A, 72 days in the case of B, 50 days in D, 57 days in V, 53 days in W, 48 days in X, and 53 days in Z. These figures are based upon the time elapsing between the date of the first positive culture and the last positive culture. Inasmuch as each of these persons probably harbored diphtheria organisms from the time of inoculation, although three of them did not show positive cultures until a few days later, it would be more nearly correct to increase the minimal period by 5 days in the case of A, and 2 days each for W and Z. Four of the seven carriers (B, D, V and Z) showed a positive culture at the time of the last examination, so that we feel reasonably sure that they continued to be carriers after that date. Two others (W and X) did not show positive cultures at the last examination, but had done so practically constantly up to that time. It is less certain but nevertheless quite probable that the last positive cultures obtained from these two persons did not measure accurately the duration of the carrier state. A was the only carrier concerning whom we feel assured that he no longer harbored diphtheria organisms at the termination of our period of observation. The probable duration of the carrier state in these seven individuals, therefore, may be tentatively estimated as follows: A 33 days, B 72 days plus, D 50 days plus, V 57 days plus, W 57 days plus, X 56 days plus and Z 55 days plus.

2. *May the lodgment and growth of the bacilli be prevented by the previous injection of diphtheria antitoxin?*

The lodgment and growth of the organisms introduced into the throat of Z were not prevented by the previous injection of antitoxin. In Y, who showed only one positive culture and that 43 days after inoculation, the development of the carrier state may have been prevented by the antitoxin which she received the day prior to inoculation. It has been pointed out, however, that A, who received no preliminary injection of antitoxin, showed only 4 positive cultures. The effect of diphtheria antitoxin, therefore, in preventing the colonization of the organisms introduced, was neither complete nor striking; from the results of our previous experiments with animals * and with human beings we think that it probably had no influence whatever in preventing the development of the carrier state.

Natural immunity against diphtheria toxin as manifested by a negative Schick test did not prevent the development of the carrier state in A, D and Z, but Y, who received a preliminary injection of antitoxin and whose skin test was also negative, showed only one positive culture. The evidence is, therefore, far from convincing that the presence of natural immunity against diphtheria toxin may prevent the lodgment and growth of diphtheria bacilli in the throat.

3. *Are such organisms, when introduced, capable of producing (a) clinical diphtheria; (b) any subjective symptoms; (c) any objective signs in the appearance of the throat?*

The four members of the group (A, D, Y and Z), who gave evidence of natural immunity against diphtheria toxin, as evidenced by a negative Schick test, two of whom (Y and Z) had this natural immunity reinforced by a preliminary injec-

* Gelien, J., Moss, W. L., and Guthrie, C. G.: The Effect of Diphtheria Antitoxin in Preventing Lodgment and Growth of the Diphtheria Bacillus in the Nasal Passages of Animals. Johns Hopkins Hosp. Bull., 1920, XXXI, 381.

tion of antitoxin, did not develop clinical diphtheria and showed no local or general effect, either objective or subjective, as the result of inoculation with virulent diphtheria bacilli from the throat of a healthy carrier.

The four persons (B, V, W and X), who had no natural antitoxic immunity as evidenced by a positive Schick test and who received no preliminary injection of antitoxin, all developed definite clinical diphtheria. From these four cases, however, we cannot say that the same result would invariably follow the introduction of virulent organisms into the throats of persons giving a positive Schick test.

4. *Will any cases of clinical diphtheria develop among the associates of artificially produced, "healthy" carriers of virulent diphtheria bacilli obtained from the throat of a healthy carrier?*

As has been pointed out, two sorts of carriers were produced among the persons in the group by the inoculation with virulent diphtheria bacilli. No cases of clinical diphtheria developed among the associates either of the healthy carriers (A, D and Z), or of the convalescent carriers (B, V, W and X).

5. *Are the organisms introduced into the throats of normal human beings in any way changed by this procedure, particularly as to (a) morphology; (b) staining characteristics; (c) cultural characteristics; (d) ability to produce toxin (virulence)?*

When isolated in pure culture after prolonged sojourn in the human throat, the bacilli were not altered in morphology or in their staining or cultural characteristics. The bacilli recovered from each of these persons throughout the period covered by our observations had retained their ability to produce toxin.

Comment

From the results of these experiments it is evident that there is no essential difference between virulent diphtheria bacilli (as determined by guinea-pig inoculation) from the throat of a healthy carrier and those from a patient with clinical diphtheria. The reliability of the animal test for toxin production has been confirmed, since it has been shown that organisms which are virulent for guinea-pigs are capable of producing diphtheria in susceptible human beings. Our earlier conclusions concerning the harmless nature of non-virulent diphtheria bacilli have received indirect confirmation since one member of the group (B), who became a carrier but suffered no other ill effect, subjective or objective, as the result of the introduction of non-virulent organisms into his throat in 1913, promptly developed clinical diphtheria following inoculation with virulent organisms in 1914. The reliability of the Schick reaction as an index of antitoxic immunity is well shown by the fact that all members of the group with a positive skin test developed diphtheria following inoculation, whereas those with a negative skin test did not.

Two questions were mentioned in the introduction which are not answered by our work. Evidently the failure of healthy carriers of virulent diphtheria bacilli to contract the disease is not because the organisms which they harbor are incapable of producing it. May this immunity be dependent upon the presence of circulating antitoxin? Information on this point is scanty, as those authors who refer to the subject at all have, for the most part, made only general statements, drawing no distinction between convalescent and healthy carriers or failing to mention whether the carriers harbored virulent or non-virulent organisms. Our own experience has not been extensive but is given, together with that of a few others, in the following table (Table IV).

TABLE IV

Results of Schick Tests

Observer	In convalescent carriers of virulent organisms	In healthy carriers of non-virulent organisms	In healthy carriers of virulent organisms	After spontaneous recovery from diphtheria
Kolmer*	Pos. or neg.	Pos. or neg.	Negative.	.....
Park†	Pos. or neg.[1]	Pos. or neg.	?[2]	Pos. or neg.[4]
Place‡	Pos. or neg.[3]	Pos. or neg.	Negative.	Pos. or neg.[5]
Guthrie, Marshall and Moss.	Pos. or neg.	Pos. or neg.	Negative.	Pos. or neg.

* Kolmer, John A., and Moshage, Emily L.: The Schick Toxin Reaction for Immunity in Diphtheria, Am. J. Dis. Child., 1915, IX, 189.
† Park, W. H.: Personal communication.
‡ Place, E. H.: Personal communication.

[1] Usually become negative within a few months after they were positive at first.
[2] Almost invariably negative but occasionally positive and in such cases the patient usually develops diphtheria again.
[3] Data insufficient but Park says he knows that a few are Schick-positive.
[4] Majority of seven cases Schick-positive.
[5] Negative immediately after recovery but may become positive later.

From Table IV, column 4, it is apparent that in many carriers of virulent diphtheria bacilli, failure to develop the disease may reasonably be attributed to adequate protection by circulating antitoxin, since they give a negative response to the Schick test. Although we have encountered no exception to this rule in our own work, the results of Park suggest that some of the healthy carriers of virulent organisms are without protection by circulating antitoxin; we feel, therefore, that a sweeping conclusion from our own small number of observations might be misleading. There may be other factors which play a part in the immunity which these carriers possess; at least such a possibility merits consideration.

Certain rather puzzling facts are known which serve to accentuate the incompleteness of our knowledge concerning diphtheria.

(1) Convalescent carriers may harbor virulent organisms for long periods after recovery from the disease, long after all passive immunity from injected antitoxin has disappeared, and show strongly positive skin tests indicating an absence of active immunity derived from circulating antitoxin developed by the carrier (Table IV, column 2).

(2) It has long been believed that individuals recovering from diphtheria without antitoxin treatment, accomplished this feat by means of the development of an active immunity, and this view is probably correct. We had supposed that this immunity was due to the elaboration of antitoxin within the body of the patient, and so were greatly surprised to learn that in persons recovering from diphtheria spontaneously, the Schick test may be positive, indicating an absence of sufficient

circulating antitoxin to prevent the recurrence of the disease (Table IV, column 5). As has been mentioned, one member of our group (W) had diphtheria at the age of six, recovered without treatment with antitoxin and showed a positive Schick test years later; that this was a true indication of susceptibility to diphtheria was proven by the fact that she developed the disease following experimental inoculation of her throat.

(3) Although it is believed that individuals possessing a natural antitoxic immunity, as manifested by a negative Schick test, are not susceptible to diphtheria, by no means all persons with a positive Schick test contract the disease even on prolonged and repeated exposure. Persons with positive Schick tests differ markedly in this respect, some never contract the disease and others have repeated attacks; so pronounced is this difference that it cannot be entirely due to a difference in exposure to infection. Another member of our group (B) was frequently exposed to diphtheria for several years, in the care of patients ill of the disease, in the study of carriers and in experimental work in the laboratory. It may be urged that there is no proof that diphtheria bacilli actually reached the nose or throat of this individual in the course of any of these procedures, but no one with much experience in taking throat cultures from children has escaped plentiful spraying from his patients. In the experimental work with animals, virulent diphtheria bacilli were introduced into the nostrils of cats, rabbits and guinea-pigs by means of a tiny swab made of a wisp of cotton on the end of a pig bristle. During the course of this work which was carried on at close range, the animals responded to each introduction of the swab with vigorous sneezing which can scarcely have failed to disseminate the bacilli and "expose" B rather thoroughly. His Schick test was positive, yet his only attack of diphtheria followed experimental inoculation with a massive dose of virulent organisms in the course of the work which forms the basis of this paper.

(4) It has been shown that the introduction of virulent diphtheria bacilli into the upper respiratory passages of several species of animals will not cause diphtheria, although the animals are susceptible to the effects of diphtheria toxin when it is injected. If the inoculation is accompanied by abrasion of the mucous membrane, however, diphtheria may develop promptly. Other paradoxical observations might be added to the list but these will suffice.

How are these apparently conflicting facts to be reconciled with each other and with the existing views concerning the immunity in diphtheria? The positive Schick test, which undoubtedly occurs sometimes in persons who recover from diphtheria without treatment with antitoxin, may possibly be explained as follows: An individual, recovering spontaneously, may do so as the result of an active immunity resulting from the elaboration of his own antitoxin. If he produces it readily and in great excess he would probably remain Schick-negative for life and not again contract the disease. He might produce it much less generously—just sufficiently to overcome the disease—have a slight excess present for only a short time after the attack and eventually show none at all. Such a person presenting a positive Schick test before the attack, would probably show a negative reaction immediately thereafter but somewhat later would again become positive. Thus, an individual after spontaneous recovery from diphtheria might present either a positive or a negative reaction, depending to a certain extent on when the test was made.

The other points mentioned—(1), (3) and (4)—are more difficult to reconcile and suggest that there may be more than one kind of immunity in diphtheria. The assumption of a local, cellular immunity seems necessary to explain the conditions mentioned above concerning diphtheria in animals. Just as animals quite susceptible to the effect of diphtheria toxin, when injected, differ from susceptible human beings in the degree of resistance of their mucous membranes, so susceptible human beings (positive Schick test) may differ from each other in the same respect. Such an immunity would go far to explain why those convalescent carriers who have a positive Schick test do not invariably and immediately have another attack. It would also offer a plausible explanation for the observation that some individuals with a positive Schick test may resist prolonged exposure to infection. If some of the healthy carriers of virulent diphtheria bacilli should be found to have positive Schick tests, it would explain their failure to develop the disease.

We are unable to say why it has been so difficult to trace cases of clinical diphtheria to healthy carriers of virulent diphtheria bacilli. As has been pointed out, it is certainly not because the organisms which they harbor are incapable of causing the disease nor can it be dependent upon natural immunity possessed by all their associates, if the immunity be limited to the presence of circulating antitoxin. Here also, the presence of a local, cellular immunity in some of the persons apparently susceptible would help to explain the facts observed. This is merely another way of saying that the number of persons in the community who are relatively insusceptible to diphtheria may be much greater than would be surmised from the results of the Schick test alone. If such an immunity could be shown to occur, it would help materially to explain the great discrepancy which exists between the number of carriers of virulent organisms and the incidence of cases of clinical diphtheria, by emphasizing the relatively slight opportunities for doing harm open to the healthy carrier of virulent diphtheria bacilli.

Above all, we would not be interpreted as attempting to question the well-established fact of antitoxic immunity or to discredit the Schick test which is a most valuable index of such immunity. It is not impossible to reconcile the facts observed with the theory advanced. The relation between toxin and antitoxin is a quantitative relation. When properly performed a negative Schick test indicates an antitoxin content of at least 1/40 to 1/30 of a unit per cubic centimeter of blood. No one thinks for a moment that, if an individual with a negative Schick test were subjected to a very large injection of toxin of high potency, his natural immunity would enable him to escape all harmful effect. In a similar way, the local defense might be overcome by the introduction of an

enormous number of organisms at one time, particularly if these were rubbed in, as was the case in our inoculations. We may carry the analogy further. By no means everyone has natural antitoxic immunity; it is quite improbable that all have any considerable local immunity; theoretically, an individual might have either, neither or both. Each type, when present, probably confers relative rather than absolute protection upon the possessor. It is possible that none of the four members of our group with positive Schick tests, who developed diphtheria following inoculation, possessed any local immunity, but if they had, one could understand how this outer defense was overwhelmed by the method of attack. All this is mere theory, however, in itself unprofitable unless followed up by experimental proof.

In a few words may be given our impression concerning the relation of the healthy carrier of virulent diphtheria bacilli to the health of the community. Although the results of our experiments indicate that the virulent organisms from the throats of healthy carriers are capable of causing clinical diphtheria when the proper opportunity is afforded, we have not obtained evidence, either from this work or from our previous studies of carriers, that diphtheria is spread by their agency under ordinary conditions. It is our belief (1) that such carriers constitute a potential menace to the health of the community but (2) that their opportunity for dissemination of the disease among their associates is quite limited owing to the relatively small number of persons who are very susceptible to infection and consequently (3) that the actual part which they play in the spread of diphtheria is probably quite small.

Conclusions

1. Virulent diphtheria bacilli present in the throats of healthy carriers are capable of producing clinical diphtheria and do not differ from those obtained from patients with the disease.

2. Virulent diphtheria bacilli retain their characteristics despite long residence in the human throat or transfer from one human being to another.

3. The guinea-pig test is a reliable index of the inherent ability of diphtheria bacilli to cause clinical diphtheria in susceptible human beings.

4. The Schick test is a reliable index of the presence or absence of antitoxic immunity against diphtheria.

5. Experimental diphtheria in human beings has a short incubation period, produces marked constitutional effects, and is accompanied by a sharp febrile reaction. It may be cured promptly by the early injection of antitoxin in adequate dosage.

THE SIGNIFICANCE OF "HEMOLYTIC INFLUENZA BACILLI"

By Arthur L. Bloomfield

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As the study of respiratory bacteriology has progressed, it has become apparent that special explanations are needed for the presence of various organisms in the upper air passages and that it is no longer possible to assume that the chance advent of a bacterium is followed by colonization and growth.[1] In another place[2] we have attempted to define the significance of the various bacteria encountered in the upper respiratory tract according to the following scheme:

A. The organism may be a member of the constant normal throat flora.

B. The organism may be an extraneous one which is present as a transient in the throat.

C. The organism may have become established in a local focus of disease in the upper air passages.

D. The organism may be one which is the cause of and is associated with the presence of an acute disease.

But aside from the above, still another possible explanation of the presence of bacteria in the upper air passages presents itself, namely, that under special conditions an organism may attain a more or less wide dissemination among groups of individuals without being primarily the cause of disease and without being localized in a definite focus of infection. Certain observations, such as the frequent presence of hemolytic streptococci[3] or meningococci[4] in the throats of contacts during epidemics of disease caused by these organisms, suggest that this may be the case, although complete demonstration of such a state of affairs is still lacking.

During the spring of 1920, while making studies of the throat flora of healthy individuals,[5] we were impressed by the almost constant finding of influenza bacilli in large numbers in practically all of these people. At the time we were inclined to regard these organisms as normal inhabitants of the pharynx, but inasmuch as there had recently been an epidemic of respiratory disease (influenza), it seemed wiser to delay judgment of their exact status. After an intermission during the summer, work was resumed in the fall of 1920 and we were surprised to find the influenza bacillus conspicuously absent in cultures made by exactly the same methods in a similar and to some extent identical group of individuals. On the other hand, a hemolytic Gram-negative hemophilic bacillus which was encountered in only two cultures in the spring of 1920 was found with almost constant uniformity in the throats both of normal people and those suffering from "colds." This observation seemed of sufficient interest to warrant following it further, inasmuch as light might thereby be thrown on general questions of the significance of bacteria found in the throat under various conditions.

We wish, therefore, to present some observations on the occurrence of "hemolytic influenza bacilli," not with the idea of contributing anything new as to the general incidence of

this organism in the upper air passages, but in order to bring out its significance in relation to that of other groups of bacteria.

THE ORGANISM

The hemolytic influenza bacillus, as studied by us, is culturally and morphologically essentially identical with the influenza bacillus save that, when grown on blood agar, a wide zone of hemolysis of the so-called beta type is produced. The colonies on this medium vary in size up to 2 mm.; they are pale greyish, rather translucent, and usually slightly sticky. The organisms are Gram-negative, and smears from 24-hour growths show long and short bacilli or thread forms. Growth is scant on human blood agar, absent in ordinary media not containing hemoglobin, and profuse on Avery's medium. This organism was described in 1919 under the name of Bacillus X by Pritchett and Stillman, whose original account may be quoted.[6] "The colony varied between 1 and 2 mm. in diameter, was clear, convex and mucoid. . . . . The bacillus is Gram-negative, takes the counter-stain deeply, and when taken from agar surface cultures appears as long, tangled threads somewhat similar to the threads often seen in pure cultures of Bacillus influenzæ. In blood-broth cultures it appears as a small fat bacillus without chain formation. It does not grow on plain sheep serum, or glucose agar.

"On blood agar the abundant growth appears as a somewhat granular, fairly heavy film, with marked hemolysis of red cells. This hemolysis is as marked as that produced by hemolytic streptococci. . . . . No pathogenicity could be demonstrated for mice, rats or rabbits." This organism was encountered by Pritchett and Stillman "in about two dozen individuals" during a series of cultures made on several hundred healthy people. They make no comment on its significance, but apparently do not regard it as etiologically related to respiratory infection. During some of the epidemics of measles in the military camps this organism was commonly present,[7] and various observers have found it from time to time in the throats of normal people. Rivers[8] reports its presence in about 30 per cent of normal men, and in a series of about 100 cultures made in the spring of 1920 we isolated it twice.

In summary, then, these hemolytic Gram-negative hemophiles, according to previous observations, are not members of the constant normal throat flora, but do occur from time to time in healthy individuals. They are non-pathogenic for animals and do not appear to be causally related to disease in man.

METHODS

A swab passed systematically over the tonsils, pharynx, and soft palate was plated on rabbit blood agar. A technique was employed which secured a good spread of colonies. The organisms were identified by colony appearance, morphology, and subculture. The number per swab was estimated as accurately as possible.

The individuals studied comprised a heterogeneous group of physicians, nurses, medical students, laboratory attendants, and hospital patients. In some cases several cultures were made, in others only a single one. "Colds" were prevalent during part of the period covered by the study and some of the subjects were affected at the time at which the cultures were obtained.

The results are summarized in the following tables:

TABLE I

INCIDENCE OF "HEMOLYTIC INFLUENZA BACILLI" IN THROAT CULTURES FROM NOVEMBER, 1920, TO AUGUST, 1921

Nov. 1–Nov. 15..	+++++++	
Nov. 16–Nov. 30.	+++++++++++++ ++++	0
Dec. 1–Dec. 15...	++++++++++++	000
Dec. 16–Dec. 31..	+++	000
Jan. 1–Jan. 15 ..	++++++++++++++ ++	00000000000000
Jan. 16–Feb. 1 ..	++++++++++++	0000000000000000000 0000000000
March	++++++	0000000
April	+++++++++	0000000000
May	+++	0000
August	++++++++++++ +++++	00000000000000000000 000000

* Each positive culture is indicated by (+) and each negative by (0)

TABLE II

NUMERICAL SUMMARY OF TABLE I

Date	Total No. cultures	Per cent positive	Total No. of people examined	Per cent positive
Nov. 1–Nov. 15	6	100	3	100
Nov. 15–Dec. 1	17	94	7	100
Dec. 1–Dec. 15.....	14	80	5	80
Dec. 15–Jan. 1.....	6	50	4	50
Jan. 1–Jan. 15.....	28	50	23	48
Jan. 15–Feb. 1.....	42	36	35	40
March	13	49	4	100
April	18	44	9	66
May	7	47	5	60
August.....	52	33	52	33

From Tables I and II it appears that hemolytic influenza bacilli were found almost uniformly in cultures made during November and December, 1920, whereas, since then, the incidence has been somewhat below 50 per cent. That the latter figure has been more or less constant for the past six months seems to indicate the present normal level. In looking for an explanation for the higher figure during the earlier cultures a possible but uncertain explanation is found in the fact that "colds" were especially prevalent at that time and that most of the cultures were made during or following an acute coryza. On the other hand, in several cases of "colds" hemolytic influenza bacilli were altogether absent.

In a further attempt to explain the presence of these organisms a series of differential cultures was made from various parts of the upper air passages. The results are summarized in Table III. It appears that in every case but one the organisms were present only sporadically and in small numbers. In Ma, on the other hand, they were constantly present in large numbers on the pharynx and both tonsils. This person had had no clinical respiratory infection during the past winter and showed no special abnormality in the upper air passages. The tonsils were small and scarred.

Discussion

Before discussing the significance of the facts brought out in the tables in regard to the occurrence of "hemolytic influenza bacilli," it is advisable to summarize certain general points. In the first place, it does not seem possible to attach any pathological significance to the presence of these organisms, at least during the past winter. The majority of individuals harboring them were clinically normal. While they were frequently present in some cases of "colds," in other instances they were persistently absent. Furthermore, the topographical cultures did not show, except in the case of Ma, a predilection for any particular part of the upper air passages—the organism was isolated from tongue, tonsils, or pharynx. In other words, a carrier state in association with a local focus of diseased tissue, such as a tonsil, cannot be assumed as a general explanation of its presence. Finally, we are dealing with an organism which is perishable outside the body and which is not an inhabitant of the skin surfaces. There is no apparent source, therefore, which would account for a frequent transient invasion as in the case of Staphylococcus albus which is readily introduced from the nose or body surface.

What then is the explanation of the presence of these organisms in about 50 per cent of a large series of throat cultures? As pointed out elsewhere,[5] there is evidence to show that certain bacteria have become adapted to a free growth on the mucous membranes of the upper air passages. It is found for example that the green-producing streptococcus, which is a member of the normal flora of adult throats, is already established in the mouths of infants 24 hours after birth. A high degree of affinity must exist here, which perhaps has developed during a long period of evolution. On the other hand, there exist bacteria which under all circumstances seem to fail to adapt themselves to growth in the upper air passages. The colon bacillus, for example, despite its ready access to the mouth is not found there in the case of clinically normal individuals. Is it possible that under special and as yet undefinable circumstances partial, abortive, or temporary adaptations of organisms to growth in the upper air passages may occur? Such an assumption would account for the wide occurrence of influenza bacilli at the time of the epidemic of 1918 and during the year following it, and for the reported instances of large numbers of temporary carriers of hemolytic streptococci and meningococci in association with epidemics of disease due to these organisms. We have also collected numerous individual observations which can be explained in no other way. Er, for example, whose throat flora had been studied at frequent intervals for over a year suddenly yielded a yeast in large numbers in the cultures. This organism had not previously been present, and there was no disease or infection at

TABLE III

Differential Cultures from Various Parts of the Upper Air Passages Showing Incidence of Hemolytic Influenza Bacilli

Patient	F.							Bl.					Er.			Ma.				
Date	Mar. 12	Mar. 14	Mar. 16	Mar. 18	Mar. 25	Apr. 12	May 13	Mar. 19	Mar. 22	Mar. 25	Apr. 11	May 15	Mar. 21	Mar. 31	Apr. 11	Apr. 25	Mar. 22	Apr. 4	Apr. 16	May 8	May 16
Nose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pharynx	0	0	0	0	0	0	many	0	0	0	0	0	0	0	0	0	several hundred	40	a few	∞	many
Right tonsil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	∞	0	many	∞	∞
Left tonsil	0	0	0	0	0	0	0	0	0	0	0	0	0	a few	0	0	many	1	many	many	60
Tongue	0	0	0	0	0	20	40	2	0	50	0	0	0	0	0	3	0	0	0	0	0

Patient	H.				D.				B.					M.		
Date	Apr. 8	Apr. 12	Apr. 19	Apr. 27	Apr. 8	Apr. 18	May 13	May 17	Apr. 13	Apr. 20	Apr. 29	May 6	May 17	Apr. 14	Apr. 28	May 6
Nose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pharynx	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0
Right tonsil	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
Left tonsil	0	0	0	0	0	0	a few	0	0	0	0	0	0	0	0	0
Tongue	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

the time of its occurrence. Cultures made almost daily showed this yeast for about ten days, after which it disappeared quite rapidly. Some sort of a biological reaction had clearly taken place—the organism could not have persisted for 10 days without colonizing, but the adaptation was not complete, and consequently elimination was the result.

In the case of the "hemolytic influenza bacilli" we are inclined to assume the above explanation. The organism is not a member of the constant habitual flora, nor was it associated with acute disease or local foci or infection except perhaps in occasional cases. There was no outside source to account for a frequent transient incidence, and yet it was present in nearly 50 per cent of cultures made more or less at random among a mixed group of people.

It seems, therefore, that here we have an instance of an organism which shows incomplete adaptation to growth on the mucous membranes of the upper air passages, although it can exist there in an abortive way without becoming predominant, and independently of the usual accessory factors which explain a carrier state of a foreign organism.

We wish, therefore, to add this possibility to the list of explanations already given of the presence of various bacteria in the throat. Studies from this point of view on the significance of pneumococci and influenza bacilli are now in progress.

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EXPERIMENTAL STUDIES ON HYDROCEPHALUS

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The effect of intravenous injections of solutions of different concentrations on the pressure of the cerebro-spinal fluid was demonstrated for the first time by Weed and McKibben (1919)[1] in their experiments on cats. They showed that such an injection of a concentrated solution of sodium chloride, of other electrolytes and of dextrose, invariably produced a marked reduction of the pressure of the cerebro-spinal fluid, the fall being often below zero. Conversely, they also found that a hypotonic solution (distilled water), given intravenously, was followed by a marked and sustained rise in the pressure of the cerebro-spinal fluid. These results were attributed to osmotic changes taking place in the body-fluids subsequent to the alteration of the salt content of the blood. A change in the actual size of the brain accompanied these alterations in the pressure of the cerebro-spinal fluid. These physiological findings were confirmed in man by Cushing and Foley (1920)[2] and by Ebaugh and Stevenson (1920).[3] Foley and Putnam (1920)[4] not only verified the observations on animals but demonstrated that the same alterations in cerebro-spinal fluid pressure could be brought about by administration of the solutions by mouth or by injection into the intestine. These observations suggested that a series of experiments be undertaken to determine the effect of similar solutions (hypertonic and hypotonic) both on brain bulk and on the pressure of the cerebro-spinal fluid in the dilated cerebral ventricles of hydrocephalic animals. If such changes in the pressure of the cerebro-spinal fluid were observed to follow the intravenous injection of a hypertonic solution it was then planned to investigate, by this means, the interesting problem of absorption of the cerebro-spinal fluid in the closed ventricles of hydrocephalus. For a marked reduction of the pressure of the cerebro-spinal fluid, under these experimental conditions, might well be interpreted to mean an increased absorption of the ventricular fluid.

METHOD OF EXPERIMENTATION

Artificial Production of Hydrocephalus.—For the success of these experiments the production of an internal hydrocephalus was essential. The typical pathological picture of this type of hydrocephalus has been successfully produced by various investigators in dogs and cats. The experimental procedure employed has concerned usually the occlusion of normal pathways of drainage of the cerebro-spinal fluid, intraventricular or extraventricular; this occlusion has been brought about by mechanical means or by the injection of different substances producing inflammatory reactions which finally blocked the pathways of the fluid. In addition to these methods the low ligation of the vein of Galen has produced different degrees of hydrocephalus.

Dandy and Blackfan (1913,[5] 1914[6]) succeeded in causing internal hydrocephalus in dogs by the occlusion of the aqueduct of Sylvius with pledgets of cotton and also by the ligation of the vein of Galen. Frazier and Peet (1914)[7] reported similar experiments with identical results, after employing similar technical procedures. Thomas (1914)[8] produced internal hydrocephalus in dogs by the intraventricular injection of aleuronate in starch, causing an inflammatory reaction that subsequently occluded the ventricular pathways. Dandy (1919),[9] under the same principle, was able to produce a communicating type of hydrocephalus by the introduction of strips of gauze, saturated with iodine, around the mesencephalon. Other workers such as Burr and MacCarthy (1900),[10] and

Flexner (1907),[10] by the injection of different irritating substances or of a suspension of micro-organisms, produced internal hydrocephalus in kittens and monkeys; in these experiments the primary pathological picture is to be considered an inflammatory meningitis, with the secondary association of the hydrocephalus.

The simple and uninvolved technique of Weed (1920)[11] for the artificial production of internal hydrocephalus was followed in these experiments. The facility with which hydrocephalus of any degree desired was produced within a short time by this method was considered advantageous for the establishment of experimental conditions. This technical procedure for the production of hydrocephalus has been employed with success in these observations, as it also was by Wislocki and Putnam (1921)[12] in studies of the absorption of the cerebro-spinal fluid from the dilated ventricles.

Two methods of equal practical advantage were introduced by Weed for the production of internal hydrocephalus; both employed injections of suspensions of lamp-black. In the first procedure the injection was made directly into the lateral ventricles, while in the second the injection was made into the subarachnoid space at the cisterna cerebello-medullaris through the occipito-atlantoid ligament. Both procedures produced in these experiments typical internal hydrocephalus in kittens, and although both methods were of equal simplicity, we agree with Weed that the subarachnoid route gave a better distension of the ventricles.

A 10 per cent suspension of lamp-black (Germantown Black) in physiological saline solution was used for the injection in all the cases. After the insertion of the sterile needle into the lateral ventricle or cisterna cerebello-medullaris, as much cerebro-spinal fluid as possible was allowed to escape. This was found necessary in order to avoid an excessive intracranial pressure when the lamp-black suspension was introduced; the escape of fluid served also as an indicator that the needle was correctly placed within the ventricle or subarachnoid space. After the release of the fluid 1 c. c. of the suspension of lamp-black was injected very slowly. In some animals, spontaneous respiration was interrupted during the latter stages of the injection; when this occurred, artificial respiration was given without delay. This cessation of respiration was especially frequent in the very young kittens, in which the injection of a cubic centimeter of fluid caused considerable increase in intracranial pressure.

The age of the kittens used for lamp-black injection in this series of experiments varied from 48 hours to 22 days. The age of the animal and the condition of ossification of the cranial vault were important factors in the rate of dilatation of the ventricles and in the degree of enlargement of the head. In very young kittens (one to four days old), diastasis of the cranial vault with distinctly palpable separation of the sutures and enlargement of the fontanelles was noticed as early as the second day after the introduction of lamp-black suspension. In older animals, however, where the union of the bony plates of the skull was advanced and firm, the increase in the intracranial tension did not produce any appreciable enlargement of the head or loosening of the cranial sutures until six or seven days from the time of the lamp-black injection. Marked hydrocephalus, with considerable enlargement of the head, was successfully produced in these kittens, results practically identical with those obtained by Weed. The symptoms of ventricular distension were of a typical character (see Fig. 1), and pronounced cases were obtained at the end of the second week after injection.

Recording of Pressure of the Cerebro-Spinal Fluid and Intravenous Injection of Solutions.—In general, the technique described by Weed and McKibben was used throughout. Ether given through a tracheal tube ensured uniform anesthesia both for the control and the hydrocephalic kittens; the method also provided a convenient means for artificial respiration when the necessity arose. The lateral ventricles were entered by an 18-gauge lumbar-puncture needle, with its obturator in place. The puncture was made by passing through the middle of the widely separated fronto-parietal suture. A calibrated U-manometer with a bore of 1 mm., filled with Ringer's solution to the zero point, was quickly attached to the puncture-needle on withdrawal of the stylet. One or more drops of cerebro-spinal fluid were invariably lost in this manipulation; replacement of this fluid by Ringer's solution was made in the manometer. Distinct pulsations of the column of cerebro-spinal fluid due to the heart-beat and respiratory movements were immediately observed in the manometer if the connections were properly adjusted and the point of the needle was free in the dilated ventricle. In these experiments the cardiac pulsations varied from a faint beating of the fluid to 2 mm. excursion, while the respiratory movements were from 2 to 5 mm., *i. e.*, the excursion between the high point reached in expiration and the low point reached in inspiration. The lower point of the respiratory excursion was taken for reading.

Injections of the solutions were all made intravenously. These were given with a syringe by way of one of the femoral veins. In every case administration of the solutions, especially of the hypertonic, was done very slowly with an average rate of 0.7 c. c. per minute.

The body temperature was carefully maintained, during the experiment, by means of an electric lamp and hood. This was of considerable importance in view of the weakened condition of the animals and the length of time required to complete the experiments, occasionally more than two hours.

Physiological Observations

Initial Pressures.—The first level reached by the cerebro-spinal fluid in the manometer, just after its adjustment, was recorded in each case. These initial readings were not considered to be accurate records of the normal pressure, however, because of the unavoidable dislocation of some of the fluid into the manometer and the loss of one or two drops of cerebro-spinal fluid in adjusting the manometer and needle. A second pressure was therefore recorded when the column of fluid attained a fairly constant level, five to ten minutes later; this was taken as the normal initial pressure. Such determinations

were found to average 4 mm. higher than the first. This slight rise of pressure in the beginning probably represents the natural restoration of the fluid displaced and lost in the adjustment of the manometer.

Observations of initial pressures were made on 12 hydrocephalic kittens and on six normal ones. The hydrocephalic kittens were examined from 4 to 20 days after the injection of lamp-black and ranged in age from 9 to 25 days. The average pressure obtained in hydrocephalic cases was 126 mm. of cerebro-spinal fluid. The maximal recorded pressure was 171 mm. and the minimal 100 mm. These pressures, when compared with similar initial readings obtained from normal kittens of approximately the same age and size, were found to be much higher, as might be expected. The maximal recorded pressure obtained in normal kittens was 102 mm., the minimal was 66 mm. and the average was 76 mm. of cerebro-spinal fluid.

Analysis of the initial pressures of the different hydrocephalic animals in regard to age and duration of the artificial lesion failed to reveal any definite relation. There was, however, noticed an important difference between the pressure of the cerebro-spinal fluid in those cases produced by intraventricular injection of lamp-black and those caused by subarachnoid injection. The initial pressure was higher in the former than in the latter, the averages being 141 mm. and 117 mm. respectively. A maximal pressure of 171 mm. and a minimal pressure of 117 mm. were found for the former and 155 mm. and 103 mm., respectively, for the latter. This difference of pressure in the two groups of cases may be explained by the mechanical effect of the lamp-black particles deposited directly against the walls of the ventricles, in those animals subjected to intraventricular injection.

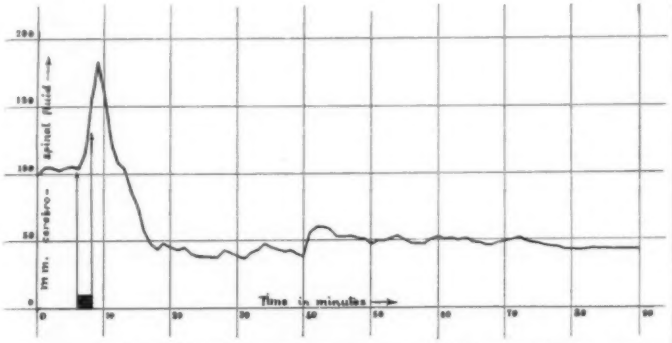

FIG. 1.—Hydrocephalic kitten No. 11. Pressure of intraventricular cerebro-spinal fluid with intravenous injection of 2 c. c. of 30 per cent sodium chloride, during blocked interval.

Hypertonic Solution.—Twelve observations of the effect of intravenous injection of hypertonic solutions upon the pressure of the cerebro-spinal fluid in hydrocephalic kittens have been made in the course of this work and the results have been uniformly constant.

Sodium chloride in 30 per cent concentration was the hypertonic solution used; 2 c. c. of the solution were given in each case. At first the dosage proved frequently fatal, but with proper precautions in the manner and rate of injection and with due regard to the general condition of the individual animal, uniformly good results were obtained.

Examination of pressures of the fluid when carefully plotted showed in general an abrupt initial rise of the pressure of from 10 to 90 mm., usually starting with the beginning of the injection. This rapid rise, in a majority of cases, continued even after the end of the injection, the duration of which was usually about two minutes. The rise was immediately followed by a rapid reduction of the pressure reaching the initial level within three or four minutes and continuing downward until it attained its lowest level. The minimal pressure was usually recorded in from 25 to 30 minutes after the completion of the injection.

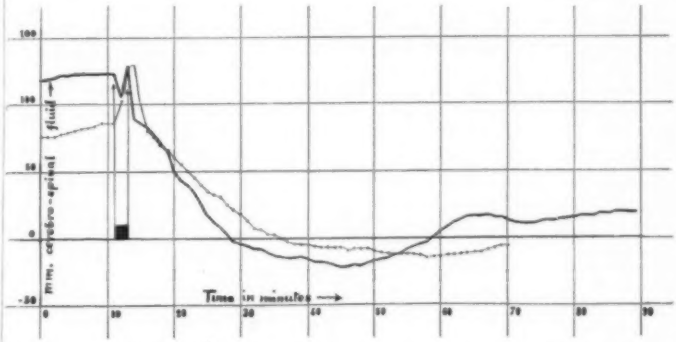

FIG. 2.—Hydrocephalic kitten No. 10 and normal control. Pressure of cerebro-spinal fluid with intravenous injection of 2 c. c. of 30 per cent sodium chloride, during blocked area.

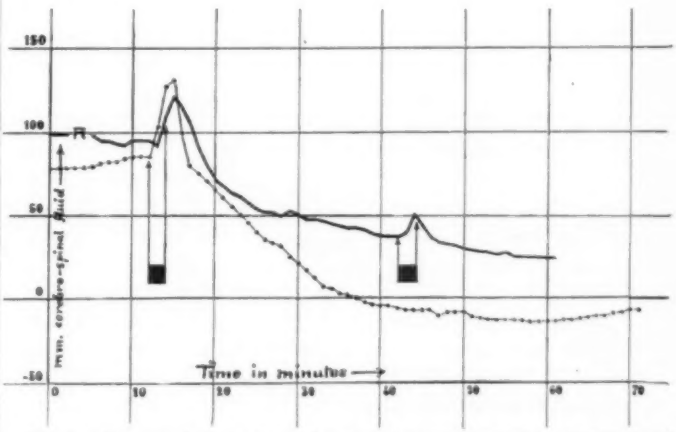

FIG. 3.—Hydrocephalic kitten No. 8 and normal control. Pressure of cerebro-spinal fluid with two separate intravenous injections of 2 c. c. of 30 per cent sodium chloride. The control received but one injection of hypertonic saline. Replacement (R) of the intraventricular fluid with solution of potassium ferrocyanide and iron ammonium citrate was carried out before the hypertonic injection in the hydrocephalic case was given.

Fig. 1 represents a typical curve showing the effect of an intravenous injection of 2 c. c. of 30 per cent NaCl upon the pressure of the intraventricular cerebro-spinal fluid in hydrocephalic kittens. A sudden rise of 87 mm. above the initial pressure was recorded in this case; this increase was soon followed by an equally rapid fall of 150 mm. The minimal

pressure obtained was only plus 37 mm. In some of the cases in this group the cerebro-spinal fluid pressure fell below zero. Fig. 2 shows the chart of one of these animals, a fairly typical curve, which reached a negative pressure of 22 mm. In this case the ascent was not as marked as usually obtained, but the drop was the lowest recorded in the entire series. In Fig. 2 the lowest pressure was reached 30 minutes after the termination of the intravenous injection of salt solution. The return to normal pressure was very gradual, consuming, as in other cases, a period of from two to three hours before reaching the initial level. With this figure is included a control tracing recorded from a normal kitten of the same litter and given an intravenous injection of an equal dose of hypertonic saline solution, the cerebro-spinal fluid pressure being recorded from the subarachnoid space. The time of the injection in the two cases was made to overlap in the plotting to afford a better comparison. In some cases a slight drop of the pressure was noticed just at the start of the intravenous injection, as shown distinctly in Fig. 2; the majority of cases, however, did not show this initial fall.

A characteristic finding in the hydrocephalic kittens subjected to this procedure was the depression of the sutures and fontanelles coincident with the drop of the cerebro-spinal fluid pressure. This was especially marked in advanced cases of hydrocephalus with considerable enlargement of the head, in which the cranial bones were widely separated. As a general rule, the lower the drop of cerebro-spinal fluid pressure, the deeper was the depression along the cranial sutures and fontanelles.

Figs. 3 and 4 are two other tracings showing the typical effect on the intraventricular cerebro-spinal fluid pressure in hydrocephalic kittens of the intravenous injection of hypertonic salt solution. The experimental animal represented by Fig. 3 received two injections of 1 c. c. each of hypertonic solution with an interval of 28 minutes between the two doses. The drop of the pressure, as noticed here, was proportionate to the size of the dose administered. It was also noticed that the effect of the second dose was not so marked as that of the first.

Hypotonic Solution.—Intravenous injection of distilled water, as the hypotonic solution, in hydrocephalic kittens gave a result similar to that obtained by Weed and McKibben in normal adult cats, *i. e.*, a marked rise of the intraventricular cerebro-spinal fluid pressure. This effect on the pressure of the cerebro-spinal fluid was repeatedly obtained in the form of a prompt and enduring rise of the pressure, which usually reached its maximum height from 20 to 30 minutes after the injection was begun. This maximum was in every case maintained for several minutes, ordinarily from 10 to 20, after which the fluid pressure began to fall very gradually, reaching the original pressure two to three hours later.

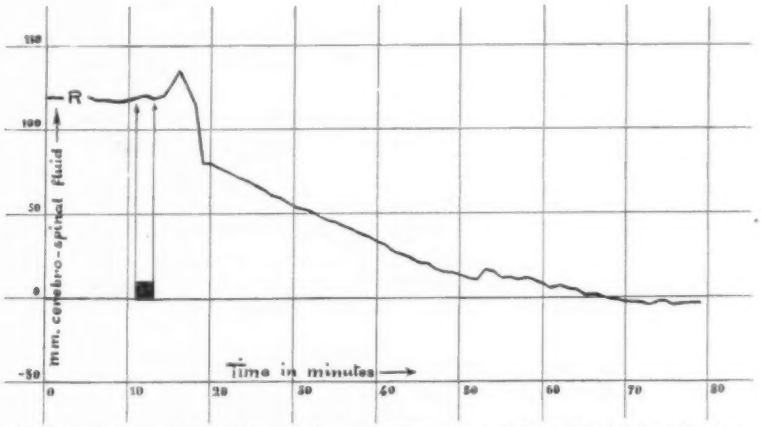

FIG. 4.—Hydrocephalic kitten No. 4. Pressure of cerebro-spinal fluid with intravenous injection of 2 c. c. of hypertonic salt solution after the replacement (R) of the intraventricular fluid with solution of potassium ferrocyanide and iron ammonium citrate.

Fig. 5 is a typical tracing of the changes in the cerebro-spinal fluid pressure following the intravenous injection of a hypotonic solution. The amount of distilled water used in this case was 20 c. c. injected very slowly into one of the femo-

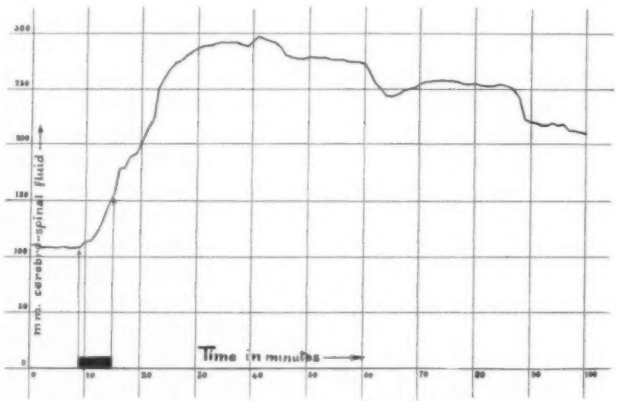

FIG. 5.—Hydrocephalic kitten No. 15. Pressure of the intraventricular cerebro-spinal fluid with intravenous injection of 20 c. c. of distilled water, during blocked interval.

ral veins. The curve reproduced is only a third of the entire observation made in this particular case. The fall was so gradual that the initial pressure of the fluid was not reached until after a period of three hours. The complete tracing in this case showed a rise in the pressure of the cerebro-spinal fluid to its maximum of 5 mm. per minute in contrast to a fall to normal at the rate of only 0.7 mm. per minute.

Fullness of the head and distension of the open sutures and fontanelles were noticed in these hydrocephalic kittens during the marked rise of the intracranial pressure following the intravenous injection of hypotonic solution.

ABSORPTION FROM THE DILATED VENTRICLES OF HYDROCEPHALIC ANIMALS

At the present time it appears to be fairly well established that the drainage of cerebro-spinal fluid is chiefly by way of the venous system and to a lesser degree also along the lymphatics. From the experimental work of previous investigators it is apparent that attention was mainly directed to the physiological aspects of the problem of absorption and that the detailed anatomical proof of the exact pathway of escape and the definite structures responsible in the absorptive processes have been but partially worked out.

Key and Retzius (1876),[12] whose important studies mark the beginning of modern work on the subject of cerebro-spinal fluid absorption, were the first to demonstrate the passage of colored gelatine, injected into the subarachnoid space, through the Pacchionian granulations into the dural sinuses. They held that cerebro-spinal fluid passed normally from the subarachnoid into the subdural spaces through the granulations and then into the sinuses.

The experiments of Weed (1914)[11] on the absorption of cerebro-spinal fluid have supported the conception of the process set forth in the monograph of Key and Retzius. With the use of solutions of potassium ferrocyanide and iron ammonium citrate and the production of the Prussian blue reaction, Weed concluded that the return of cerebro-spinal fluid from the subarachnoid space to the general circulation is principally by a process of filtration through the arachnoid villi into the great venous sinuses of the meninges. He also agreed that there is an accessory drainage of the cerebro-spinal fluid from the subarachnoid spaces into the lymphatic system.

The results of the observations conducted in man by Dandy and Blackfan (1914)[6] on the absorption of the cerebro-spinal fluid in internal hydrocephalus (obstruction of the channel of exit into the subarachnoid space) suggested clearly that a slow absorption takes place in the cerebral ventricles. They injected phenolsulphonephthalein into the cerebro-spinal fluid and by its quantitative output in the urine they could determine the rate of absorption of the dye from the ventricles. In all the seven cases with completely occluded ventricles that were under their observation the phenolsulphonephthalein was detected in the urine 20 to 45 minutes after the intraventricular injection of this indicator. This result was definite evidence of absorption of the cerebro-spinal fluid from the occluded ventricles of hydrocephalus. They found that the rate of absorption from the ventricles in these cases was from 1 to 2 per cent in two hours; that is, up to 2 per cent of the injected drug was excreted in the urine within the first two hours. In the extensive work of these two investigators they, however, repeatedly asserted that in obstructive hydrocephalus practically no absorption takes place from the ventricles.

The recent experiments of Wislocki and Putnam (1921)[13] on the intraventricular absorption of the cerebro-spinal fluid in hydrocephalic kittens constituted an important step in the solution of the problem of the exact course of escape of the ventricular fluid. For the replacement of the cerebro-spinal fluid in the dilated ventricles, they employed trypan blue and solutions of potassium ferrocyanide and iron ammonium citrate as used by Weed for the Prussian blue reaction. With these two means for identification of the exact course taken in the drainage of the ventricular fluid they were able to demonstrate, anatomically, that absorption occurred to some extent from the ventricles in hydrocephalic animals and that the pathway of escape was through the ependyma into the intercellular spaces and finally into the perivascular spaces. This was a clear anatomical demonstration of intraventricular absorption of the cerebro-spinal fluid in hydrocephalus.

The main object of the following observations was the determination, anatomically, of the structures responsible in the absorption of the intraventricular cerebro-spinal fluid. The physiological proof that such absorption actually occurred was presented in the preceding sections of this report. It is the hope that in this paper are combined the physiological and anatomical studies tending to give clear evidence of this intraventricular absorption of the cerebro-spinal fluid.

Two principal series of observations were undertaken on hydrocephalic kittens to determine the absorptive processes taking place intraventricularly. Similarly, two identical groups of experiments were afterward performed on normal kittens to serve as control and to elucidate further some of the findings in the observations on the hydrocephalic cases. One series of experiments provided for the intraventricular substitution of the cerebro-spinal fluid by a solution of equal parts of 1 per cent each of potassium ferrocyanide and iron ammonium citrate; the substitution of this foreign solution in hydrocephalic and normal kittens was followed quickly by the intravenous injection of hypertonic salt solution. The other series of hydrocephalic and normal kittens was likewise subjected to the intraventricular substitution of the cerebro-spinal fluid by the same foreign solution; then, after letting the animals live for two hours, they were killed without any administration of the hypertonic solution. In both cases all the specimens were soon preserved by aortic injection of fixing fluid consisting of 10 per cent formalin with 1 per cent hydrochloric acid. The addition of the mineral acid caused prompt precipitation of Prussian blue granules *in situ*, giving exact evidence by the distribution of the granules of the course taken by the true solution. Gross and microscopic examinations of the specimens were then made.

The substitution of the cerebro-spinal fluid in hydrocephalic kittens was done by determining the initial pressure of the intraventricular fluid with a manometer connected with the puncture needle. Once the initial pressure was recorded, the manometer was detached from the needle and all the cerebro-spinal fluid was allowed to escape. The volume of the released fluid was determined and then exactly the same amount of the ferrocyanide and citrate solution was introduced. The introduction of the solution was done with a graduated syringe connected with the same puncture needle. The manometer was again inserted and the height of the fluid observed. If the initial pressure was not reached, more of the solution was introduced into the manometer until the original level of the fluid was attained.

The hypertonic solution was given through one of the femoral veins and a dose of 2 c. c. of 30 per cent solution of sodium chloride was usually administered.

In animals in which the hypertonic solution was injected after the replacement of the fluid, a sufficient time was allowed for the reduction of the pressure of the cerebro-spinal fluid, as indicated in the manometer, before the animal was sacrificed. This observation of the physiological reaction permitted one to be certain that absorption had already taken place in the ventricles. For the very same reason, those subjects which were not given injections of the hypertonic solution after the substitution of the intraventricular fluid with the ferrocyanide and citrate solution were allowed to live two hours before they were sacrificed.

In the experiments on normal kittens no observation of initial pressure was made. The substitution of the fluid was performed by the introduction of puncture needles of the same caliber into each of the lateral ventricles. One of these, the more dependent, was used for the escape of the fluid; while the other, the more elevated, was employed for the introduction of potassium ferrocyanide and iron ammonium citrate solution. The introduction of this solution was done by gravity, by means of a small glass funnel connected with a rubber tubing to the higher puncture needle. Very little pressure was used in introducing the solution in this way and free escape of any fluid under abnormal tension in the normal ventricles was assured. This method of replacement did not permit any increase in the normal intraventricular tension. The injection of hypertonic solution in normal kittens was made through the femoral vein and the same dose of salt was administered in all cases.

The use of the Prussian blue reaction with the solution of potassium ferrocyanide and iron ammonium citrate in this experiment was considered to be advantageous: the procedure is the only feasible method that can be used with accuracy in this kind of investigation. It was Weiss (1879)[18] who apparently first introduced this method of precipitating *in situ* such foreign fluids, and since his time many have taken advantage of this reaction in solving the varied problems of anatomy and physiology. Weed has emphasized the manifold advantages of this method over other preparations as a means of studying the processes of absorption of true solutions elsewhere in the body. The precipitation of ferric ferrocyanide (Prussian blue) from the dual solution is effected by the mineral acid combined with the formalin fixative. This precipitate is not affected by the ordinary technique of staining and preparation of histological sections; the characteristic finely granular, blue precipitate can be easily identified under the microscope.

Hydrocephalic Cases with Replacement and Hypertonic Injection.—Macroscopic findings: Gross sections of the encephalon from all of the seven hydrocephalic animals in this group showed the presence of a marked degree of dilatation of the ventricles and thinning of the cerebral cortex. In cases where the hydrocephalus was produced by intraventricular injection of lamp-black the entire ventricles were dark in color throughout except the choroid plexuses which very frequently remained quite free from the lamp-black particles. Animals which received the lamp-black suspension in the cisterna cerebello-medullaris had the ventricles only partially darkened by the black granules.

The distribution of the Prussian blue precipitate was very characteristic in these hydrocephalic kittens and a description of the distribution in one will suffice for all the cases. On section of the specimen, the blue precipitate appeared quite dense in the dilated ventricles, massed against the ependyma wall, and penetrating in zones of diminishing intensity the brain substance. The blue precipitate was most abundant upon and just beneath the lining ependyma of the walls of the lateral ventricles. It gradually became less and less marked towards the periphery or away from the ependyma. The inner grey matter of the brain substance appeared to be the only portion colored with the precipitate; the white cortex was free throughout from the blue coloration. In the region of the basal ganglia and especially within the substance and around the basal attachment of the septum pellucidum the blue precipitate of ferric ferrocyanide was the most abundant. (See Figs. I and II.) The ependyma throughout was also stained blue, though not as intensely as the grey substance just beneath it. An important finding macroscopically was the faint coloration of the choroid plexus with the blue; these structures stood out in contrast to the dark pigment of the surrounding walls.

The wall of the fourth ventricle in some cases was also covered with blue precipitate especially in those cases where the initial injection for the artificial production of hydrocephalus had been made into the cerebello-medullary cistern. The aqueduct of Sylvius was patent in these animals. In three of the seven cases the vein of Galen was found to possess in its lumen some Prussian blue precipitate. In no other situation outside of the ventricles was the ferric ferrocyanide precipitate recognizable.

Microscopic findings: Blocks of brain tissue were taken from each specimen under observation; these were dehydrated in graded alcohols, embedded in celloidin, sectioned and stained for microscopic examination. Stained sections from different regions of the wall of the dilated ventricles were carefully examined to determine the distribution of the ferric

ferrocyanide precipitate and to obtain thereby information regarding the pathway of escape of the ventricular fluid. The ventricular surface of the ependyma was covered here and there with fine blue granules and also with coarser lampblack particles. (See Fig. III.) Prussian blue in the form of very fine granules was found within the cytoplasm of the ependymal cells and to a certain extent also in between them. The blue precipitate was however most abundant in the tissue beneath the ependymal lining. Under higher magnification these foreign particles were found lying against the external surface of the endothelial wall of the blood capillaries. Very fine precipitates were distinguishable inside the minute capillaries located just beneath the ependymal epithelium. In some of the cases these blood capillaries were so full of this precipitate that the course of the capillary networks could be actually followed by studying the distribution of these granules. (See Figs. III and V.) Farther away from the ependyma the larger vessels did not all show blue precipitate in their lumina. Under closer examination the vessels which possessed blue granules were identified as veins; the arteries were free from the precipitate. It is worthy of note that the veins located in the septum pellucidum and in the ventricular surface of the basal ganglia were in all cases filled with Prussian blue.

These findings give some clue as to the probable course of exit of the ventricular fluid. In connection with the observation of blue granules in the veins it may be remembered that the venae septi pellucidi, usually two in number, empty directly into the small cerebral veins together with the vena terminalis and the vena choroidea. The vena terminalis comes directly from the ventricular surface of the basal ganglia.

In no specimen from this series of animals was the blue precipitate found inside the epithelium of the choroid plexuses; nor were any of the blue granules found inside the capillaries of the choroid plexuses nor in the larger vessels going into or coming out from them. Some precipitate was found over the surface of the choroid epithelium or between the adjoining villi, but never inside the covering epithelial cells. The finding of the precipitate on the outside of the high columnar cells of the plexus was to be expected, but the failure to discover any blue granules within the cell cytoplasm or beneath the cells indicates definitely that no absorption of fluid occurred through these structures.

Hydrocephalic Cases with Replacement Only.—Macroscopic findings: Practically the same gross anatomical features were found in all these cases as encountered in the hydrocephalic animals which had been given hypertonic saline, after the replacement of the ventricular fluid with ferrocyanide and citrate solution. Fig. IV typically represents a horizontal section of the encephalon of one of the cases. It will be noticed that the Prussian blue precipitate is most abundant beneath the ependymal lining. The grey substance of the occipital and frontal lobes was especially filled with dense precipitate. In many cases the septum pellucidum, especially its basal attachment, and the ventricular surface of the basal ganglia were abundantly filled with the blue granules. Such findings coincide fairly well with the distribution of the Prussian blue in the series given hypertonic solution.

Microscopic findings: The cellular protoplasm of the ependymal cells showed the presence of fine blue granules of ferric ferrocyanide. These granules were scattered here and there in the cytoplasm although a greater number were located near the base of the individual epithelial cells. The endothelial walls of the blood capillaries just beneath the ependyma and also to a certain distance away from it were covered with the blue precipitate. Farther from the lining epithelium of the ventricles the small veins could be distinguished from the arteries not only by their morphology but by the presence of the iron precipitate inside their endothelial wall. No Prussian blue granules could be identified within the cytoplasm of the covering cells of the choroid plexuses, nor in the stroma or capillaries beneath these cells. This microscopic picture of the distribution of the blue granules in this series was identical with that found in the first group, with the only minor difference that the amount of Prussian blue found beneath the ependyma, in the grey substance, was less in this series. (See Fig. V.) This indicates that under these experimental conditions the process of absorption was not as active as under the influence of the hypertonic solution introduced into the blood stream.

Normal Cases with Replacement and Hypertonic Injection.—Macroscopic findings: Gross sections of the brains of this control series were made and examined in the same manner as in the previous cases. The normal slit-like ventricles of the brain were clearly outlined by the distribution of the Prussian blue precipitate. The blue tint invaded the brain substance to a distance of 3 to 7 mm. away from the ependyma, gradually becoming fainter towards the cortex. The ependymal lining of the ventricles was colored blue. This normal lining epithelium appeared to be much less prominent than the ependyma of the dilated ventricles in hydrocephalus. The latter was thicker and showed a clear demarcation line from the underlying tissue unlike this normal ependyma, the thickness of which was hardly recognizable with the naked eye. The wall of the fourth ventricle was also stained blue, although not so intensely as that of the lateral and third ventricles. The septum pellucidum and the basal ganglia bordering the ventricles were all colored with the precipitate of ferric ferrocyanide. (See Fig. VI.)

Microscopic finding: The cytoplasm of the lining ependyma showed the blue granular precipitate. Some of the ferrocyanide and citrate solution was precipitated inside the ventricles and the granules were found lying directly against the surface of the ependyma. The blood capillaries beneath it showed the presence in their lumina and against their endothelial walls of the blue precipitate. Farther away from the ependyma in the cerebral grey substance these granules were less in number and were only found in the smallest veins. In the substance of the septum pellucidum and in the portion of the basal ganglia adjacent to the ventricles a considerable amount of the iron precipitate was likewise encountered both

in the cells of the ependyma covering them and in the network of capillaries in their substance.

Normal Cases with Replacement Only.—Macroscopic findings: The same distribution of the Prussian blue precipitate was met with in this normal series as in the hydrocephalic and normal animals already described. The blue tint was localized in the grey substance adjacent to the ventricular ependyma, and especially in the substance of the septum pellucidum and in the basal ganglia. The wall of the fourth ventricle was also colored blue. The apparent difference existing between this and the normal animals which were given injections of hypertonic saline was the far less extensive penetration of the colored solution into the tissues in this present series.

Microscopic findings: The ependymal cells and the underlying capillaries were, as in the previous cases, filled with blue granular precipitate. The number of these granules was, however, considerably less than in the preceding normal series; the penetration of the foreign solution into the cerebral grey substance was also less extensive. The cells of the choroid plexuses were wholly free from the Prussian blue granules.

The gross and microscopic findings in the two groups of normal animals (after replacement of the cerebro-spinal fluid in the ventricles with the solution of potassium ferrocyanide and iron ammonium citrate), one with the intraventricular injection of hypertonic solution and the other without the saline administration, coincided fairly closely with the findings in the two hydrocephalic series. The penetration and distribution of the precipitated ferric ferrocyanide were similar in the two series, but a lesser penetration or absorption of the injected solution was apparent in those animals, hydrocephalic or normal, which were not given an injection of the strongly hypertonic solution.

Discussion

The increased intracranial tension of the cerebro-spinal fluid in hydrocephalus has been known for many years. Many accounts of the intraventricular fluid spurting from the needle on tapping appear in the literature, but many of these statements appear to be exaggerated. Although it is evident that there exists an increased pressure of the cerebro-spinal fluid in hydrocephalus, it is present only in a limited degree. The findings in this study indicate a difference in pressures of the cerebro-spinal fluid in the hydrocephalic and normal control animals, but this difference, while marked, is not extraordinary. The maximal recorded cerebro-spinal fluid pressure obtained in this series of observations was 171 mm.; the average of all was 126 mm. and the lowest was 100 mm. As might be expected the cerebro-spinal fluid pressure of hydrocephalic kittens was higher than the normal pressures obtained from control kittens. The average pressure for the normal kittens in this study was 76 mm., the highest recorded was 102 mm. and the minimal pressure was 66 mm. There existed, therefore, a difference in pressure of 50 mm. of cerebro-spinal fluid between the hydrocephalic and normal kittens.

Compared with the normal pressure of the cerebro-spinal fluid obtained by Weed and McKibben in their experiments on adult cats, the average pressure of the control kittens was lower, while that of the hydrocephalic kittens was much higher. Weed and McKibben gave an average of 119 mm., a maximum of 155 mm. and a minimum of 90 mm. Foley and Putnam[4] gave more variable normal cerebro-spinal fluid pressures for cats with an average of 127 mm., a highest pressure of 255 mm. and a lowest of 65 mm. These determinations of cerebro-spinal fluid pressures of adult cats, when compared with the pressures found in normal kittens, are higher.

The marked dilatation of the cerebral lateral ventricles in the hydrocephalic kittens should be considered as the primary and direct effect of the augmented intraventricular pressure of the cerebro-spinal fluid. This increased pressure within the ventricles is in turn the inevitable result of the damming-back of the fluid into the ventricles, caused by the obliteration of the exits for the intraventricular fluid into the subarachnoid space. These factors and their subsequent results have been discussed by Weed (1920)[3] in his paper on the experimental production of hydrocephalus.

One of the most striking results of the increased dimensions of the lateral ventricles was the extreme thinning of the cerebral cortex. In Fig. I it will be noticed that this process of rarefication took place more in the occipital and parietal regions of the hemispheres than elsewhere, indicating that these are the points of lesser resistance to the internal pressure. The temporal, as seen in Fig. II, and then the frontal regions were the next areas to yield to the increasing pressure of the intraventricular fluid. The basal nuclei appeared to be the least affected in the compression of the ventricular walls. In all specimens the outlines of these nuclei were wholly visible in the floor of the lateral ventricles. Weed described this picture of the dilatation of the ventricles and thinning of the cerebral cortex as closely approximating a partial reversion to the embryonic type of cerebral ventricle.

As the chief pathway of return of cerebro-spinal fluid to the blood stream is outward through the subarachnoid space, it is to be expected that in this artificial production of hydrocephalus there must exist a lack of a normal absorption-rate of the cerebro-spinal fluid. Under these experimental conditions with a normal rate of elaboration of the fluid from the choroid plexuses of the ventricles and with the pathway of escape of the fluid into the subarachnoid space occluded, the normal absorption through the meningeal channels becomes insignificant. Assuming that absorption through the ependymal lining of the ventricles takes place (as demonstrated in these experiments), the normal balance between secretion and absorption of the fluid cannot be maintained, as the subarachnoid pathways of escape are necessary to care for all the fluid produced. The maintenance of a normal equilibrium between the formation and the absorption of the cerebro-spinal fluid requires the patency of the communications between the ventricle and the subarachnoid space.

It should be remembered in this connection that the choroid plexuses have been found in these experiments to be free from

lamp-black granules and from the precipitate of ferric ferrocyanide, both over their lining secretory epithelium and in their substance; it seems more than probable that they are secreting at a normal or slightly reduced rate in these hydrocephalic animals. These observations suggested that the choroid plexus is possessed of only one function,—the elaboration of the cerebro-spinal fluid. There is no evidence that the choroid plexuses take part in the absorption of the fluid from the ventricles.

One of the earliest demonstrations of a slow intraventricular absorption of the cerebro-spinal fluid was presented by Dandy and Blackfan (1914)[9] in the obstructive type of internal hydrocephalus. The method used by them in this experimental work was the introduction of phenolsulphonephthalein into the occluded ventricles and later identification of this substance in the urine. They found that in from 20 to 45 minutes after the introduction of this dye stuff into the closed ventricles, it could be detected in the urine. This finding indicated an absorption of the cerebro-spinal fluid from the occluded ventricles of hydrocephalus. This physiological observation received anatomical support when Wislocki and Putnam (1921)[12] reported somewhat similar experiments in hydrocephalic kittens. In their observations, they made intraventricular injections of trypan blue and the solution of potassium ferrocyanide and iron ammonium citrate. With the microscopic identification of these solutions after fixation, the probable pathway of escape of the intraventricular fluid could be studied; these workers for the first time demonstrated, anatomically, that absorption occurs in hydrocephalic animals through the ependymal lining of the ventricles.

Experiments similar to those of Wislocki and Putnam have been many times repeated in this investigation. The anatomical findings of Wislocki and Putnam showing evidence of intraventricular absorption have not only been definitely substantiated in this work, but a clear physiological proof has also been demonstrated for the first time both in hydrocephalic and normal animals.

Our experiments, in which substitution of the ventricular fluid by an isotonic solution of potassium ferrocyanide and iron ammonium citrate was carried out in hydrocephalic and normal kittens, give additional anatomical evidence of the pathway of exit of the cerebro-spinal fluid from the ventricles. Again, our observations on the alteration of the pressure of cerebro-spinal fluid in hydrocephalic kittens by the intravenous injection of solutions of different concentration offers physiological evidence of an intraventricular drainage of cerebro-spinal fluid.

Gross and microscopic examination of the brains of the experimental animals amply demonstrated that absorption does take place through the ependyma and that the pathway is into the underlying capillaries with drainage into the smaller and then larger venous channels. Such a course of drainage was much more easily made out in those subjects given intravenous injection of hypertonic solution after the replacement of the ventricular fluid.

The manifest drop of the intraventricular pressure of cerebro-spinal fluid in hydrocephalic kittens following the intravenous injection of hypertonic solution of sodium chloride indicates that absorption of the fluid from the ventricle must have taken place. The marked drop of pressure of 100 mm. of cerebro-spinal fluid in many instances in our series of experiments cannot be explained alone by the physical influence of tissue contraction subsequent to the withdrawal of the tissue-fluid. The markedly thinned nervous tissue forming the wall of the dilated ventricles, cerebral cortex and medullary substance, does not seem to have sufficient volume to cause by its shrinkage the marked fall in the pressure of the intraventricular fluid in such hydrocephalic animals. The shrinkage of the brain substance, reported by Weed and McKibben,[10] while marked, hardly seems in these hydrocephalic animals the determining factor in the production of the low and occasionally negative pressures of the cerebro-spinal fluid.

The only plausible explanation for the fall of the cerebro-spinal fluid pressure following intravenous administration of hypertonic saline solution is that of a fairly rapid absorption of the cerebro-spinal fluid from the ventricles through the ependyma into the blood capillary bed. That this absorption does take place by this route is proven by the anatomical findings after the substitution of intraventricular fluid. That this absorption is more rapid after the administration of the hypertonic solution is again supported by the anatomical evidence from the series of experiments in hydrocephalic and normal kittens with replacement of cerebro-spinal fluid by the ferrocyanide and citrate solution, for the colored Prussian blue precipitate has a wider distribution and is more intense in those animals given the injection of hypertonic solution than in the controls. The increased area of the dilated ventricular wall covered by ependyma permits considerable absorption of the fluid under these circumstances; the result of this absorption is apparently shown by the great reduction of the intraventricular pressure, even to the negative values.

Another point of interest brought forth in this series of observations is that absorption of the intraventricular fluid takes place through the ependyma not only in hydrocephalic but also in normal kittens. The experiments conducted in normal kittens with the replacement of cerebro-spinal fluid in the ventricles by the ferrocyanide and citrate solution revealed the fact that slight absorption through the ependyma takes place. It should be mentioned here that a comparative study of the microscopic picture of distribution and abundance of Prussian blue precipitate in the different series of experiments suggested that absorption through the ependyma seems to be rather a slow process unless aided by the intravenous administration of hypertonic salt solution. In the normal animal, also, such intraventricular absorption, while existent, must be of almost minimal physiological importance.

The marked elevation in the pressure of the intraventricular fluid following the administration of hypotonic solution to hydrocephalic kittens was possibly due to the increased volume of the fluid and not to the increased volume of the brain. In the artificially produced hydrocephalus, the choroid plexus

was found free from lamp-black particles and in those cases with replacement of the ventricular fluid by the ferrocyanide and citrate solution no evidence of absorption of the foreign solution by the plexuses was found. Under the influence of the large injection of the hypotonic solution and its accompanying osmotic changes, the apparently normal choroid epithelium may have permitted the passage of more fluid into the ventricle than ordinarily occurs, or fluid may have passed through the ependymal wall into the ventricle. The highly differentiated character of the choroidal epithelium would probably be more efficient in preventing this fluid-passage than would the ependyma, but these hypotheses must remain speculative, inasmuch as no anatomical evidence of the process is available. Another explanation that might be ventured for the rise of the intraventricular pressure is the occurrence of rapid osmotic changes between tissue and blood, leading to the imbibition of fluid by the former with resultant swelling of the brain substance and associated increased intraventricular pressure. The markedly thin cerebral cortex forming the wall of the dilated ventricles cannot in all probability exert much influence in raising the cerebro-spinal fluid pressure to 300 mm. of the fluid as recorded in one of our observations.

From the results in hydrocephalic kittens after the intravenous injection of hypertonic and hypotonic solutions in this series of experiments, it may be concluded that the cerebrospinal fluid in the dilated ventricles may be acted upon by the alterations in salt concentration of the blood, and that the volume of the intraventricular fluid may be apparently increased or diminished by the administration of solutions of different concentrations.

Summary and Conclusions

1. The pressure of cerebro-spinal fluid in kittens in which an internal hydrocephalus has been experimentally produced was considerably higher than that of normal kittens; an average difference of 50 mm. of cerebro-spinal fluid was found in this series of observations.

2. Intravenous injection of a strongly hypertonic solution of sodium chloride in hydrocephalic animals produced a brief initial rise in the pressure of the intraventricular cerebrospinal fluid followed immediately by a marked depression. This decrease in pressure at times produced negative values. This phenomenon is probably to be explained by the apparently rapid absorption of the cerebro-spinal fluid from the dilated cerebral ventricles.

3. Intravenous injection of hypotonic solution (distilled water) in hydrocephalic kittens was invariably followed by a marked and sustained increase in the pressure of the cerebrospinal fluid. This result was possibly due to a rapid elaboration of fluid by the choroid plexuses or to an increased transudation of fluid through the ventricular walls.

4. Intraventricular absorption of cerebro-spinal fluid took place in these hydrocephalic kittens; the pathway of escape was through the ependyma into the underlying capillary network. This absorption of cerebro-spinal fluid was hastened by the intravenous administration of strongly hypertonic solutions.

5. Absorption of the cerebro-spinal fluid was similarly found taking place through the ventricular ependyma in normal kittens. The pathway of escape was the same as described above; the process of absorption was very slow in the normal animal but was hastened by the administration of hypertonic solutions. The physiological significance of this intraventricular absorption in the normal animal is probably minimal.

6. The choroid plexuses took absolutely no part in the intraventricular absorption of the cerebro-spinal fluid.

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Description of Plate

Fig. I.—Transverse section of the brain of hydrocephalic kitten No. 8, 22 days after intraventricular injection of 1 c. c. of a 10 per cent suspension of lamp-black. Replacement of the intraventricular cerebro-spinal fluid with solution of potassium ferrocyanide and iron ammonium citrate was followed by intravenous injection of hypertonic salt solution. The black stippling shows the distribution and extent of absorption of the true solution of ferrocyanide and citrate that has been precipitated *in situ*, by the action of mineral acid, as ferric ferrocyanide (Prussian blue). The pressure observation of the cerebro-spinal fluid following the injection of hypertonic salt solution in this kitten is given in text Fig. 3.

Fig. II.—A transverse section of the brain of hydrocephalic kitten No. 4, 13 days after intraventricular injection of 1 c. c. of a 10 per cent suspension of lamp-black. Replacement of intraventricular fluid with the solution of potassium ferrocyanide and iron ammonium citrate was followed by an intravenous injection of hypertonic solution of NaCl. The distribution of the Prussian blue precipitate is shown by the black stippling. The fall of the cerebrospinal fluid pressure after the hypertonic administration is shown in text Fig. 4.

Fig. III.—Drawing under high power of a section through the ependyma and underlying grey substance of the brain from hydrocephalic kitten No. 8. Replacement of intraventricular fluid with ferrocyanide and citrate solution was followed by intravenous injection of hypertonic saline. On the surface of the ependyma are large clumps of lamp-black particles and finer granules of Prussian blue

FIG. I.

FIG. II.

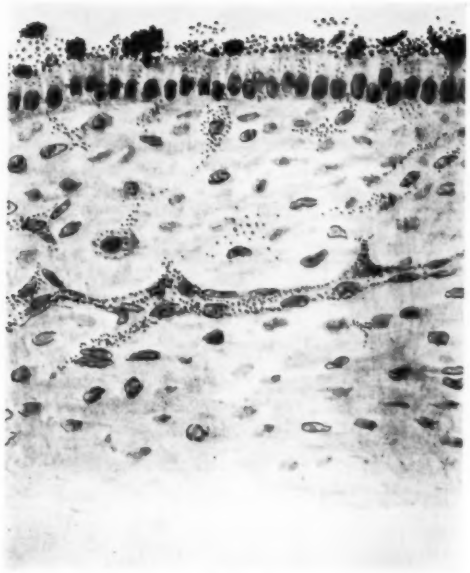

FIG. III.

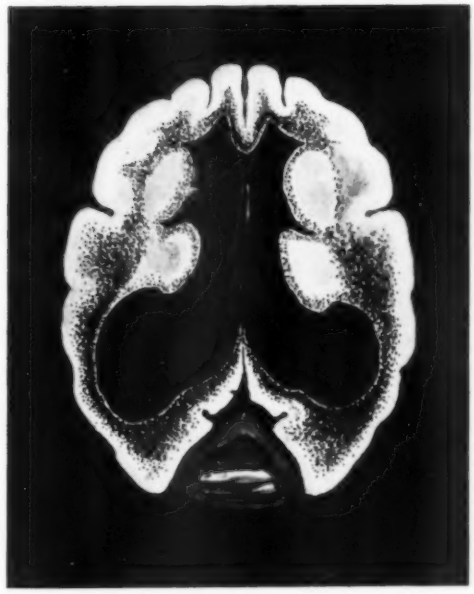

FIG. IV.

FIG. V.

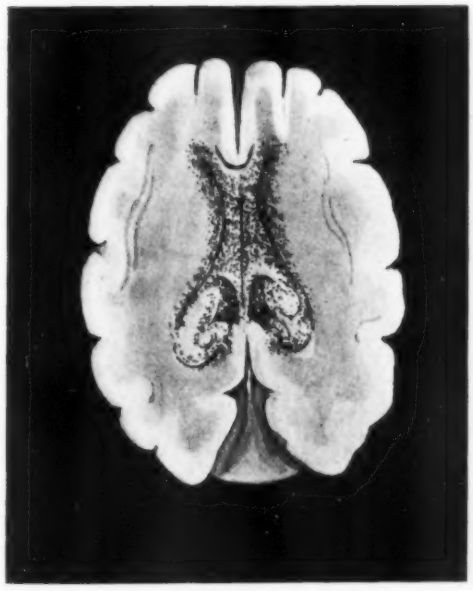

FIG. VI.

precipitate. In the epithelial cytoplasm are seen many fine granules of Prussian blue. The blood capillaries in the grey substance beneath the ependyma are filled with granular precipitate. The larger capillary vessel lower down is full of Prussian blue granules in its lumen.

FIG. IV.—Section of the brain of hydrocephalic kitten No. 11, eight days after the subarachnoid injection of 1 c. c. of a 10 per cent lampblack suspension. Replacement of ventricular fluid with ferrocyanide and citrate solution was carried out and after two hours the animal was killed without any administration of hypertonic solution. The distribution of the precipitate of ferric ferrocyanide in the grey substance is shown by the stippling.

FIG. V.—Drawing under high power of a section through the ventricular wall of the hydrocephalic kitten No. 11. Replacement of intraventricular fluid with potassium ferrocyanide and iron ammonium citrate solution was carried out but without any administration of hypertonic solution of salt. The fine granular precipitate of Prussian blue is found both on the surface and inside the cytoplasm of the ependymal cells. It is present also in the lumina of the blood capillaries of the grey substance under the ependymal epithelium. The penetration of the true solution of ferrocyanide and citrate is less extensive in this case than in the microscopic section shown in Fig. III taken from the hydrocephalic kitten No. 8.

FIG. VI.—Transverse section of the brain of a normal control kitten that had replacement of the intraventricular fluid with solution of potassium ferrocyanide and iron ammonium citrate, followed by intravenous injection of hypertonic solution. The precipitate of ferric ferrocyanide from the foreign solution is found throughout the ventricular walls; it has passed the ependymal lining, has reached the grey substance beneath the ependyma and is within the blood capillaries.

SULPHÆMOGLOBINÆMIA

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The presence of persistent and intense cyanosis which cannot be explained by cardio-vascular or pulmonary disease, by the ingestion of drugs or by the inhalation of gases, is very rare. The first example of this type of case was reported by Stokvis [1] in 1902 under the caption "Enterogenous Cyanosis." The blood of his patient was examined indirectly by applying a spectroscope to the living tissues and a narrow band was seen in the red between the "C" and "D" lines, near the orange, in addition to the usual bands of oxyhæmoglobin. The patient was suffering from severe and prolonged diarrhœa which Stokvis believed to be causally related to the production of the abnormal hæmoglobin derivative.

In the same year Talma [2] reported three other examples of the condition and confirmed the spectroscopic results obtained by Stokvis. In addition he demonstrated by direct examination of the blood serum and of solutions of the blood corpuscles that the hæmoglobin compound was intra-corpuscular and not free in the serum.

Each of these observers believed that the spectra which they had observed were dependent on the presence of methæmoglobin in the circulating blood. Although this assumption was not supported by sufficient evidence, as will be pointed out below, we are, nevertheless, indebted to them for calling attention to this rare type of cyanosis.

Van den Bergh,[3] in 1905, had an opportunity to study two cases of enterogenous cyanosis. The first patient was suffering from a rectal stricture with resulting retention of fæces; the second from a severe chronic diarrhœa. Both showed distinct cyanosis. By careful measurements of the spectra of their blood and by the use of the ammonium sulphide test,* as well as by comparison with the spectra of solutions of artificially prepared methæmoglobin and sulphæmoglobin, he proved that the abnormal pigment in the blood of the first patient was sulphæmoglobin, whereas that in the blood of the second was methæmoglobin.

These results were of fundamental importance, since they demonstrated clearly that two affections easily distinguishable by simple methods may have been included previously under the term "Enterogenous Cyanosis." This designation, therefore, should be abandoned and in its place the terms "Methæmoglobinæmia" and "Sulphæmoglobinæmia" should be substituted according to the nature of the abnormal hæmoglobin compound.

Methæmoglobinæmia occurs not infrequently after the ingestion of certain drugs. It is rare, however, in association with chronic dysentery, as in the cases reported by Stokvis and Talma and in a later case reported by Gibson and Douglas.[4] The associated disease will not be discussed in this paper as we shall limit our report to sulphæmoglobinæmia.

The records of 12 cases of the later disease, in which the diagnosis was supported by sufficient evidence, were found in the medical literature and have been summarized in the accompanying table. A case reported by Jameison [5] and one by Talma [2] possibly belong here, but since definite evidence is lacking, they will not be included. Our present case, therefore, is the thirteenth proved instance of sulphæmoglobinæmia which has been recorded:

A white man 28 years old, a draftsman by occupation, was admitted to The Johns Hopkins Hospital (Med. No. 44858), January 10, 1921, complaining of attacks of weakness during which he turned blue. His father, two sisters and a brother had died of tuberculosis within a few months before his birth. As a child he had had diphtheria, scarlet fever, mumps and measles. At the age of 11 years he was in a hospital for 14 months with hip trouble from which he recovered completely. Since that time he had been well except for rather frequent headaches. He had not suffered from constipation.

His present illness began insidiously in May, 1920, with a sensation of heaviness in the epigastrium, accompanied by weakness and exhaustion. This "all gone feeling" occurred every two to three days and lasted several hours each time. During a more severe attack, in October, 1920, his lips and nails became blue and he suffered from

* This test, apparently not previously known by van den Bergh was described in detail in "The Spectroscope in Medicine," London, 1880, by McMunn.

palpitation and breathlessness. Since that time he has had numerous attacks similar in character but of variable intensity. During the more severe attacks he became very weak and fell but did not lose consciousness. They lasted from two to eight hours and were usually preceded by severe headache and accompanied by trembling, palpitation, marked cyanosis and a peculiar sensation behind the sternum.

Physical examination was made on admission while an attack was receding. There was tremor of the hands and the patient was restless and apprehensive. The nails, lips, tongue, nose and ears and to a less extent the skin in general had a peculiar purple tint as though the patient were under a mercury-vapor lamp. The scleræ were slightly yellow. The tonsils were large, succulent and inflamed. Cardiac dulness extended 10 cm. to the left and 4 cm. to the right of the midline. The sounds were normal and no murmurs were present. There was a slight sinus arrhythmia. The lungs were clear. The fingers were not clubbed. The spleen was just palpable at the costal margin.

Laboratory Examinations.—The erythrocytes numbered 3,584,000 and the leucocytes 13,300 per c.mm. The hæmoglobin was 68% (Sahli). A fresh film showed moderate secondary anæmia and a slight leucocytosis. A differential count was normal. The fragility test showed hemolysis beginning at 0.42% NaCl and complete at 0.32% NaCl. There were 66 vitally stained erythrocytes per thousand. Roentgenograms of the thorax and gastro-intestinal tract were reported normal. The Wassermann test with the blood was negative. Several specimens of fæces showed no abnormalities. The urine showed very strong tests for urobilin at every examination but was otherwise normal. Hemolytic streptococci were obtained in culture from the tonsillar crypts.

Blood drawn during an attack was deep purple-brown in color. On spectroscopic examination of the blood diluted with distilled water, the characteristic band of sulphæmoglobin was present in addition to the bands of oxyhæmoglobin. The spectrum was identical with that of a solution of artificially prepared sulphæmoglobin and slightly different from that of methæmoglobin. Addition of a dilute solution of ammonium sulphide to various dilutions of the patient's blood caused no change in the appearance of the band in the red. That band, however, shifted slightly to the right when illuminating gas was passed through the solution. The patient's serum contained no sulphæmoglobin, and when added to various dilutions of normal red cells or laked red cells it did not convert oxyhæmoglobin into reduced hæmoglobin or sulphæmoglobin, even after long periods of incubation.

The urine and saliva contained no more nitrites than normal and no nitroso-bacilli could be grown from the latter on the medium employed by Wallis.[6]

Treatment.—The tonsils were removed and the patient was given a milk diet and frequent purgation. Bulgara tablets were administered in an attempt to change the intestinal flora. No improvement was noted when the patient was last seen (February 14, 1921).

Clinical Features

A review of the clinical histories of the reported cases is of interest since it shows that the symptomatology has been very similar in each instance. The age of the patients at the time the diagnosis was definitely established varied from 9 to 67 years. However, all but three were past 20 and under 45 years of age. All except two were females. The patients had complained, in most instances, of a variety of symptoms before they recognized the blue color of their nails or lips or suffered from the characteristic attacks. These symptoms included nervousness, weakness, palpitation of the heart, headache and occasionally periods of nausea, vomiting and severe abdominal pain. A few patients had become chronic invalids and after exploratory operation had passed from hospital to hospital for treatment. Constipation was observed in the majority of patients, and in one instance the condition was apparently cured by eradication of a rectal stricture. However, this symptom has been overemphasized since in a number of instances it was not present.

The duration of the vague symptoms enumerated above before the presence of cyanosis was recognized varied from a few months to 12 years. It is difficult, therefore, to judge whether they were dependent on mild degrees of sulphæmoglobinæmia or were symptoms of some other malady. However, they preceded the development of the characteristic attacks so constantly that we believe they may be looked upon as part of the symptom complex.

After the cyanosis had been present for some time the syndrome was usually augmented by the appearance of the more *characteristic attacks* which may be described as follows: After a severe headache of variable duration the patient complains of a peculiar "all gone" sensation behind the sternum. Palpitation, weakness, giddiness and nervousness are present and there may be some dyspnœa. The patient may fall or may actually lose consciousness. His face becomes very cyanotic and he may seem moribund. In some instances the pulse and respiratory rates have been rapid, while in others they have been very slow. The attacks vary in duration and in severity but usually last from two to eight hours. They are followed rather suddenly by amelioration of symptoms and gradually by diminution of the cyanosis.

The intervals between attacks were usually comparatively free from symptoms although some degree of cyanosis persisted and certain vague complaints were present.

Physical abnormalities were seldom observed. The fingers were clubbed in a few instances and occasionally the spleen was palpable. In one case a rectal stricture was present.

Slight secondary anæmia was present rather frequently, usually associated with urobilinuria. The fæces were normal in every instance.

The diagnosis of sulphæmoglobinæmia can be made with certainty by spectroscopic examination as follows:

A small glass container with flat sides about 1 cm. apart is partly filled with distilled water. Whole blood is added to this drop by drop until the two bands of oxyhæmoglobin in the green of the spectrum become very distinct. At this point a definite dark band in the red near the orange (between Frauenhofer lines "C" and "D") will be visible if sulphæmoglobin or methæmoglobin are present in moderate amounts. Otherwise, blood is added until the spectrum, with the exception of the red, orange and yellow, is absorbed. If no band is visible in the red at this point, the diagnosis is not possible by spectroscopic methods.

Unless a large, accurately calibrated spectroscope is used, the ammoium sulphide test must be relied upon to differentiate methæmoglobin from sulphæmoglobin. This test is performed as follows: To the spectroscopic cell containing a dilute solution of blood which shows a band in the red near the orange, a dilute solution of $(NH_4)_2S$ is added drop by drop while the spectrum is observed. If the band in the red persists after a few drops have been added, it is due to sulphæmoglobin. If it disappears, it is due to methæmoglobin.

Another confirmatory test consists in allowing acid-free carbon monoxide to bubble through the diluted solution of blood. If the band in the red and the two bands in the green shift slightly to

the right, sulphæmoglobin is present. If they remain unaltered, methæmoglobin is present.

The blood serum and urine should also be examined for the presence of abnormal hæmoglobin derivatives, since sulphæmoglobin is found only in the red blood cells, in contrast to methæmoglobin which may be found in the serum. Moreover, sulphæmoglobinuria is unknown, whereas methæmoglobinuria following drug poisoning is occasionally observed.

PATHOGENESIS

The mechanism of the production of sulphæmoglobinæmia in man is not known at present, although various observers have established certain facts which have clarified the problem to some extent. Their results will be discussed, therefore, in an attempt to bring the experimental work up to date.

It was recognized by all investigators that the condition was not dependent on the ingestion of drugs or chemicals and that in all probability it was independent of the type of food consumed. Consequently, the cause was sought in some abnormal reaction within the body. Since in many instances constipation was a prominent symptom (and one patient was cured after treatment of a rectal stricture [2]) studies were made to determine whether increased quantities of hydrogen sulphide or an increased amount of hydrogen-sulphide-producing bacteria were present in the bowel. The results of such investigations were not convincing and further attempts to explain the malady by increased production of hydrogen sulphide in the alimentary tract were abandoned.

The suggestion of Steensma [10] that the presence of nitrites in the blood might be the basis of methæmoglobin production was apparently proved by van den Bergh and Gutterink [11] for methæmoglobinæmia. Clarke and Hurtley [7] later demonstrated that the addition of powerful reducing agents, such as nitrites, to blood also greatly accelerated the production of sulphæmoglobin in the presence of minute traces of hydrogen sulphide. Wallis [6] therefore followed these leads in an attempt to elucidate the pathogenesis of sulphæmoglobinæmia. He investigated the saliva of four patients suffering from this condition and in each instance found a nitrite-producing bacillus and increased amounts of nitrites. He demonstrated, furthermore, that the serum of each patient contained a reducing body capable of converting oxyhæmoglobin into reduced hæmoglobin. He assumed, therefore, that the malady was dependent on the absorption by the blood of nitrites from the buccal cavity and of small amounts of hydrogen sulphide from the gut.

This theory is attractive, although the results obtained by Wallis could not be confirmed by Long and Spriggs [12] nor by us, and even Wallis [6] noted that the reducing agent in the blood of his patients seemed more powerful than nitrites.

The problem of the method of the production of sulphæmoglobinæmia in man, therefore, is not solved, although the evidence at hand supports, to some extent, the opinion of van den Bergh that sulphides, formed in the bowel in some manner, gain access to the blood.

The actual amount of sulphide as sulphæmoglobin in the blood of a patient with sulphæmoglobinæmia is not known, but must be very small, since West and Clarke [5] showed that 0.00003 gm. of hydrogen sulphide in 10 c. c. of a mixture of whole blood and water was sufficient to produce a definite spectrum of sulphæmoglobin. This quantity is far below the amount that can be detected by chemical methods, although Meyer [9] had shown that these were 10 times more delicate than spectroscopic tests in demonstrating sulphides in the blood of experimentally poisoned animals. These statements, of course, need confirmation. In sulphæmoglobinæmia in man, however, it is probable not only that minute traces of sulphide are sufficient to produce the abnormal pigment, but also that the sulphides are so firmly secured by hæmoglobin that pas-

Reported by	Reference	Age	Sex	Cyanosis		Spectra	Test with NH_4 2 S
				Duration	Type		
v. d. Bergh	Deutsch. Arch. f. klin. Med., 1905, LXXXIII, 86.	9	M	Several years	Constant but variable.	Band in the Red	Band persisted.
West and Clarke	Lancet, 1907, I, 272	37	F	Two years	Constant	Band in the Red	Band persisted.
v. d. Bergh and Gutterink.	Berl. klin. Woch., 1906 XLIII, 7.	67	F		Marked	Band in the Red	Band persisted.
		60	F		Marked	Band in the Red	Band persisted.
		17	F		Marked	Band in the Red	Band persisted.
Clarke and Curtis	Med. Record, 1910, LXXVIII, 987.	24	F	Few months	Constant but variable.	Band in the Red	Band persisted.
Davis	Lancet, 1912, II, 1145.	27	F	Eleven years	Constant but variable.	Band in the Red	Band persisted.
Long and Spriggs	Quart. Jour. Med., 1917-18, XI, 102.	32	F	Two years	Constant but variable.	Band in the Red	Band persisted.
Wallis	Quart. Jour. Med., 1913-14, VII, 73.	27	F	Three years	Constant but variable.	Band in the Red	Band persisted.
		37	F	Four years	Constant but variable.	Band in the Red	Band persisted.
Wynter	Proc. Roy. Soc. Med., Clin. Sec., 1908, I, 48-197.	45	F	Twelve years	Constant but variable.	Band in the Red	Band persisted.
Russell and Leathes.	Lancet, 1907, I, 659.	30	F	Eight years	Constant but variable.	Band in the Red	Band persisted.
Authors' case		27	M	Two months	Constant but variable.	Band in the Red	Band persisted.

sage of oxygen or carbon dioxide through the blood does not displace them.

The relation of sulphæmoglobin production to the oxygen carrying power of the hæmoglobin has not been determined in man nor in animals, although experiments are being undertaken to elucidate that problem. It seems probable that anoxæmia is partly responsible for the symptoms presented by the patients, although, as is well known, warm-blooded animals die from inhalation of hydrogen sulphide without the production of determinable amounts of sulphæmoglobin.

A few experiments on warm-blooded and cold-blooded animals have been undertaken and may be briefly described here. Lewin [13] administered Schlipp's salt (a compound readily liberating H_2S) to dogs by mouth, subcutaneously, or intravenously and was able to produce the spectrum of sulphæmoglobin. Meyer [9] also succeeded in producing the characteristic spectrum in the blood of a rabbit poisoned by rectal administration of H_2S. The inhalation of hydrogen sulphide by frogs is quickly followed by the formation of sulphæmoglobinæmia. These experiments have thrown little light on the possible mechanism of spontaneous sulphæmoglobinæmia in man.

Treatment

Frequent purging is recommended by all observers and this seems to have relieved the symptoms in some instances. Van den Bergh's patient was cured by operative removal of a rectal stricture. Wallis [6] speaks of cure after vaccine therapy, killed cultures of a nitroso-bacillus being employed. In the majority of instances treatment has failed to afford permanent relief and even the temporary amelioration occasionally observed may have been dependent on rest in the hospital.

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MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES ON THE MUSCULATURE OF THE MATURE GRAAFIAN FOLLICLE OF THE SOW

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In the recent literature on the morphology of the ovary, passing mention is made of the occurrence of smooth muscle fibers in the wall of the Graafian follicle. The work reported in this paper was undertaken to verify or disprove these observations, and, if this muscle were proven to exist, to determine the types of autonomic nerve fibers to its cells, and further to investigate the importance of this element in the physiological activity of the follicle.

The authors wish to express to Dr. Corner, of the Department of Anatomy, their appreciation for his generous interest and direction, without which these researches could not have been performed. We also wish to thank Professor Abel and Dr. Macht of the Department of Pharmacology for their advice and help.

History

In the initial volume of his famous journal (1849), *Die Zeitschrift für Wissenschaftliche Zoologie,* von Kölliker was the first to mention smooth muscle as a structural constituent of the ovary. In 1861 C. Aeby by means of the then much relied upon acetic and nitric acid technique made extensive investigations on the character and location of the muscle fibers in the ovaries of various birds and mammals. Two years later Grohe described the complete course of the muscle fibers in the ovary of the pig, from its hilus to the very edge of the

mature follicles, which he found it to encircle.[1] Grohe also mentioned the unusually generous distribution of smooth muscle fibers about the blood vessels of the ovary. In this he supported the hypothesis formulated by Rouget seven years earlier, that, in contracting, the muscle fibers of the ovary compressed the blood vessels, in consequence of which the congestion from impaired venous return led to the rupture of the mature follicles. For a half century little progress was made in either the histology or physiology of the smooth muscle of the ovary; more refined methods of staining were developed but nothing of note was learned. In 1909 H. von Winiwarter and Sainmont announced the occurrence of what they believed to be true smooth muscle fibers in the theca externa of the Graafian follicles of the cat. In the latter part of 1919 Thompson, in his paper on the human Graafian follicle, dwelt at length on the occurrence and purposes of muscle fibers in the theca externa, which he was not able to demonstrate very clearly by a microphotograph. He obtained his best differentiation with safranin and light green. This author stated that the muscular element could not be demonstrated in all of his preparations. Corner, earlier in the same year, briefly mentions the application of the van Gieson technique in distinguishing these fibers. Other than this, the authors have been unable to find mention of the occurrence of muscle in Graafian follicles.

The nerve supply of the Graafian follicle has been a problem long and bitterly disputed. It was early recognized by Frankenhäuser ('67) and Waldeyer ('70) that sympathetic nerves entered the hilus of the ovary with the ovarian artery. This observation was substantiated by all subsequent workers. Riese ('91) showed that the nerve fibers on entering the ovary were separated into two groups—one group accompanying the blood vessels, and the other going directly to the follicles. The workers in this field were soon divided into three camps: Riese ('91), von Herff ('92), von Gawronsky ('94), Winterhalter ('96), Brill ('15) and others believed that nerve fibers independent of the perivascular network penetrate all the layers of the follicle wall, the theca externa, the theca interna and the granulosa. The second group represented by Abel and McIlroy ('12-'13) claimed that only the two outermost coats of the follicle wall have a nerve supply. The third group which numbered among its protagonists Retzius ('93), de Vos ('94), and Mandl ('95) held that the follicle is not in any way penetrated by nerve fibers, other than those of the perivascular network.

Material

Our selection of the sow was based on several considerations. The sow has been found to ovulate spontaneously and to have an œstrous cycle of 21 days. The large size of the ripe follicles (5 to 10 mm. in diameter) offered great advantages, particularly in the physiological work. The existence of two pork-packing houses in close proximity to this laboratory assured us twice daily a supply of fresh tissue. Then too, the experience of Dr. Corner made his advice invaluable as to selection of the ovaries of animals in heat, and the rejection of pathological material.

Our tissue was obtained immediately after slaughter. The ovaries of animals that were in œstrus or just about to enter œstrus formed the chief source of our material. The œstrous period could be determined by the large size of the follicles, the retrogressing corpora lutea, the great vascularity of the peritubal tissue, and the relative pallor of the uteri. Corner ('19), in a study of the follicles of animals known to have been in heat when killed, stated that these follicles possessed clear, translucent, almost spherical walls, protruding a great part of their bulk from the ovary, as is characteristic of the species; they all measured about 7 mm. in diameter, the measurement ranging from 6 to 8 mm. in some which were distorted by crowding. The surface presented no "stigma" or other sign of impending rupture.

The Musculature of the Graafian Follicle

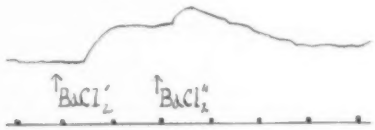
The ovaries used in studying the muscular morphology were promptly immersed in Bouin's fluid. After the Bouin fixation, and the usual paraffin embedding technique, the tissue was sectioned from 3.3 to 5 micra, either by the ordinary microtome technique, or by Professor Huber's wet knife method. The sections were then stained in hæmotoxylin with iron alum as a mordant. After decolorization, they were subjected to the van Gieson technique, for the differentiation of smooth muscle and connective tissue fibers. This stain was found to be entirely satisfactory—the bright red connective tissue and the yellow muscle fibers, with their typical nuclei, forming a brilliant contrast.

In the thecæ externæ of all our specially stained preparations, we were able to distinguish typical smooth muscle cells, with their characteristic cytoplasm and nuclei, which in their distribution gave the suggestion of a lamina over the two internal layers of the follicle, in many places incomplete and interrupted by areas of connective tissue and blood vessels. In many of our sections muscle fibers were found (Figs. 1 and 2), grouped into bundles of rather impressive proportions in juxtaposition to the cells of the theca interna. In many areas these bundles formed pure bands of muscle more than a half-dozen cells in thickness, completely devoid of intercellular connective tissue. In some places, however, only very small scattered groups of muscle cells were present in the follicular wall. Here, as has been noted in the ovarian stroma, these muscle bands seemed to be particularly numerous in the region of the blood vessels. In all of the material sectioned there was no suggestion of the existence of muscle cells in the theca interna. In sections made of a freshly ruptured follicle (the eggs were recovered from the tubes, and two normal, still unruptured, follicles were found in the ovary), the arrangement of the muscle fibers suggests that they played an active rôle in the rupture of the follicle. That the theca externa did not remain as a mere passive membrane on the periphery of the

[1] It is interesting to note that at this time, prior to the great contributions of His ('65) and Waldeyer ('70), the follicular layers had been imperfectly differentiated, the two external layers of the wall of the follicle being generally referred to as the theca folliculi.

newly ruptured follicle, but in conjunction with the other layers projected finger-like processes into the recently contracted follicular cavity, is made evident by Fig. 2. Corner ('19) has shown by means of Weigert's elastic tissue stain, that with the exception of the blood vessel walls the follicle is completely devoid of elastic tissue. Since in this species only about 25 per cent of the recently ruptured follicles contain any blood in their cavities, perhaps, as he suggests, the muscle here by compressing the wall plays the mechanical rôle of a hemostat.

Having followed the advice of Gaskell to found our issue on a firm anatomical basis before resorting to confirmatory physiological measures, we proceeded to prove the existence of muscle by functional tests. We followed the usual "in vitro" technique in this phase of our work. While the ovaries were in Locke's solution needles and thread were passed through opposite poles of the follicle, then by pulling on these sutures we were able to cut the projecting portion of the follicular wall free from the stroma of the ovary. Great care was exercised so that none of the stroma was included in the tissue removed. This bit of living tissue was suspended in a bath of oxygenated Locke's solution maintained at body temperature. The suture



Graph A.—Contraction of wall of Graafian follicle due to stimulation with barium chloride. Time intervals 10 minutes.

$BaCl_2'$ Barium chloride 1-10,000.
$BaCl_2''$ Barium chloride 1-10,000.

on the one end of the follicle was tied to a glass rod well submerged in the solution, while that on the other end was attached to a very sensitive writing lever. In this way accurate records of the muscular contractions of the wall of the follicle could be obtained on a smoked drum. At times in order to make these records more decisive we combined strips of tissue from two follicles of the same ovary.

We first made use of a solution of barium chloride, the classical means of chemically stimulating smooth muscle regardless of its innervation, with which we obtained most active contractions. Even the addition of 2 c. c. of a 0.5 per cent solution of barium chloride to 25 c. c. of Locke answered admirably for this purpose. A relatively long latent period and a gradual protracted "rise" marked the contractions obtained with this drug. With solutions of greater concentrations more marked contractions were obtained than the one recorded in Graph A. At this point we became interested in determining whether the stimulation by barium was due to its toxic effect alone. We found that very definite contractions of the tissue from the wall of the follicle could be obtained by using solutions of barium, made isotonic with Locke's solution, sufficiently dilute to permit the life of amœbæ, paramecia, and rotifers for more than 6 hours. The records which were obtained through barium stimulation were typical of smooth muscle and as barium has not been found to cause the contraction of any tissue other than muscle, we may conclude that the wall of the Graafian follicle contains non-striated muscle-fibers.

The Nerves of the Graafian Follicle

It is very difficult to stain satisfactorily the nerves of the Graafian follicle. The delicacy of the fibrils makes microtome sections of this material quite unsatisfactory, for at best the sectioned nerve fibers look like a series of fine dots of doubtful structure. It was apparent to us therefore early in our study, that the information we sought could best be obtained from gross material suitably stained. The Golgi and the Bielschowski impregnation methods gave unsuccessful results in our hands, probably because of the thickness of the follicle wall and the impermeability of the outer layers of the ovary. Good results were obtained by Ehrlich's methylene blue staining, following the modifications of J. G. Wilson. Injections of the ovary, through the ovarian or uterine arteries, with a 1/20 per cent methylene blue solution were made under great pressure, which was necessary to insure a complete injection of the mature follicles. After the tissue had been exposed to the oxidation of the air for from one-half to one hour (the time required to stain the nerves distinctly) some of the specimens were fixed in ammonium molybdate, and others in ammonium picrate. The picrate fixation made the specimens easier to tease apart and had the additional advantage of staining the muscle fibers yellow. On the other hand, the tissue after ammonium molybdate fixation cleared much better, and the specimens were made quite firm by dehydration. In specimens fixed in this way it was possible to peel off the various layers of the follicle and study them separately.

In the theca externa, the muscle-containing layer of the follicle, there is a rich plexus of nerve fibers (Fig. 4). The nerves run (10 to 15 together) in good sized non-medullated bundles, independent of the perivascular network which is also demonstrable in our preparations. These bundles of non-medullated nerve fibers give off numerous fibrils to the surrounding tissue. The fibrils have the ordinary morphology of the non-medullated nerve. As Fig. 3B shows, the nerve fibrils course among the muscle cells, running parallel with some cells and crossing others. They give a definite impression of bearing a functional relation to the muscle cells of the theca. These nerve fibrils terminate on muscle cells in sympathetic motor endings (Fig. 3A), the smaller endings covering one muscle cell, while the larger terminations overlie two or three cells. As Fig. 3A shows, these endings are of two distinct types—one ending is large, pale, clear and oval; while the other is small, dark, full of granules and triangular in form.

We have therefore a complete path: nerve fibrils running intimately among muscle fibers, sympathetic motor-endings on muscle cells, and muscle cells capable of functioning. It seems unquestionable that there must be some physiological application for this apparatus. We also thought it desirable to obtain exact information as to the type of autonomic innervation of the ovary and its follicles, since in the abundant

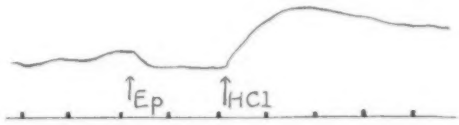
literature on the nerves of this organ we have found no mention of such a study. Thompson ('19) records some pharmacological studies made by Gunn at the former's suggestion, to demonstrate by experimental means the smooth muscle of the ovary. In one experiment conducted on the whole ovary of a virgin rabbit, Gunn elicited a response from stimulation with epinephrine (1-200,000)—an observation which led him to maintain that the ovary has true sympathetic innervation. (It is interesting to note, however, that Gunn finds the true sympathetic nerves to be excitatory nerves of the ovary in the rabbit, while we have found them to be inhibitory nerves in the sow's ovary.) In 1909 under the direction of Von Winiwarter the ovary of the cat was stimulated chemically and electrically but with negative results. Langley and Anderson ('96), in the cat and rabbit, and Kuntz ('19), in the dog, have shown through dissections and physiological degeneration experi-

GRAPH B.—Contraction of the wall of the Graafian follicle due to changes in the hydrogen-ion concentration, caused by the addition of hydrochloric acid and sodium hydroxide.

HCl' Hydrochloric acid, 0.2 c.c. of 0.3 per cent to 25 c.c. of Locke's solution.

ments that the gonads are innervated by rami from the third, fourth, fifth, and very rarely from the sixth lumbar prevertebral sympathetic ganglia. We have verified this for the sow in a dissection of a large embryo prepared in formalin. However, the important work of Gaskell, Langley and Dickinson, has indisputably shown that the only reliable method of studying autonomic innervation is by means of drugs. By such studies one is enabled to differentiate sympathetic (thoracolumbar) from para-sympathetic (bulbar-sacral) and inhibitory from excitatory fibers. We, therefore, undertook such studies on the nerve supply of the mature Graafian follicle. In this work we followed the ordinary "in vitro" technique already described in this paper. Before the work had progressed far, we noticed that the smooth muscle of the follicle wall is very sensitive to any variation of the hydrogen-ion concentration either to the alkaline or acid side. The addition of such a minute amount as 0.2 c.c. of 0.3 per cent HCl to 25 c.c. of Locke's solution gave the first rise reproduced in Graph B. It was therefore imperative to use neutral salts of the alkaloids or, if no neutral salts were available, to make the solutions neutral with $NaHCO_3$.

On the addition of pure epinephrine, furnished by Dr. Abel, a definite relaxation of the follicle was recorded (Graph C).

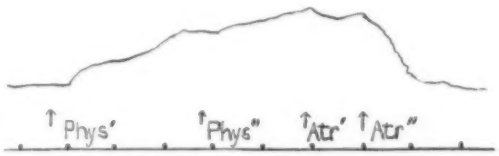


GRAPH C.—Relaxation of wall of the Graafian follicle due to stimulation with epinephrine and contraction due to the addition of acid.

Ep Epinephrine 1-5000.
HCl Hydrochloric acid.

The extent of the relaxation was practically the same with a 1-10,000 solution as with a 1-2,000,000, the amount of relaxation diminishing slightly with the increased dilution. Thus the nervous mechanism of the Graafian follicle is shown to respond to the action of a drug which stimulates fibers of the true sympathetic system. In this case the action of the drug causes relaxation of the smooth muscle fibers.

Through the addition of physostigmine sulphate (eserine) and atropine sulphate we showed that the follicle also has para-sympathetic innervation. A 1-10,000 solution of physostigmine caused a very marked contraction of the tissue (Graph D). The proof of a para-sympathetic innervation was further substantiated by the action of atropine, which paralyzed the myoneural junction of the para-sympathetic nerve fibers, so that on the addition of physostigmine no contraction was elicited; this drug also relaxed the tissue previously contracted by physostigmine (Graph D). It is clear then that the follicle has a para-sympathetic innervation, these fibers acting as contractors of the follicle wall. Similar results in all respects were obtained by us with experiments on the ovarian stroma—those drugs which stimulate the sympathetic system acting as inhibitors and the para-sympathetic stimulants contracting the ovarian musculature. All



GRAPH D.—Contraction of wall of Graafian follicle due to stimulation with physostigmine sulphate and subsequent relaxation on the addition of atropine sulphate.

Phys'	Physostigmine sulphate	1-10,000.
Phys"	Physostigmine sulphate	1-10,000.
Atro'	Atropine sulphate	1-1500.
Atro"	Atropine sulphate	1-1500.

of our results demonstrate that the ovary and the follicles of the sow have an innervation analogous to that of the intestines.

All the physiological work reported in this paper was done with the same apparatus, and yet there were marked variations

in the results obtained. With some ovaries the contractions were relatively great; while with others only feeble contractions were noted. From our observations we think perhaps that the amplitude of the contraction depends on the exact moment in the œstrous cycle at which the animals were slaughtered. Those ovaries containing large distended follicles giving the impression of imminent rupture, produced the greatest contractions, while those specimens less advanced in the cycle generally seemed to give contractions of much less magnitude.

Observations Relative to the Method of Rupture of the Follicle

Since the early part of the last century various speculations have been made as to the method of rupture of the Graafian follicle. Suffice it to say that the theories have been almost as numerous as the theorizers. Thompson in 1919 was the first to suggest that the smooth muscle in the wall of the follicle plays a major rôle in this process. It is of interest to note that it had been maintained, as early as 1859, by Pflüger that the peristaltic movements of the frog's ovaries caused rupture of the follicles.

When we found with what readiness the smooth muscle of the Graafian follicle of the sow could be stimulated to contract in vitro, we had high hopes of producing these contractions, with the ovaries still attached to the excised uteri, and thus perhaps induce rupture. We adopted several methods of procedure with the isolated organs—injections of barium chloride, physostigmine, etc., were made into the uterine and ovarian arteries, in some of which high pressure (350 mm. of Hg.) was produced by a hydrostatic apparatus, while in others a low pressure (21 mm. of Hg.) was maintained for a long period of time. Separate follicles were excised from the ovary and direct injections were made into the follicular cavity by means of a hypodermic syringe. Experiments were also performed in which a constant low head of pressure was maintained in these excised follicles, which were immersed in warm oxygenated solutions.

The results of all of these experiments were either negative or so inconstant, because of the experimental difficulties in carrying out these procedures in excised organs some time after death, that we were unable to produce any conclusive evidence as to the rôle of muscle in the rupture of the follicle. We feel confident that experiments conducted on this problem in the living animal would give fruitful results. We believe that our experiments on this phase of the problem have definitely shown that the pure mechanical hypothesis of Hensen which has been so widely accepted, that rupture is induced solely through increased arterial tension, is false. This theory is supported by a single case of rupture induced by Clark ('00) in a human ovary during an intravascular injection of carmine gelatin. In connection with our work 72 injections of the ovarian and uterine arteries of ovaries with mature follicles were made under great pressure. The follicular vessels could be seen to wash out clearly but rupture did not occur in a single instance, even though one braced himself against a wall and pushed the piston of the injection syringe with all of the physical strength available. A constant head of pressure of over 300 mm. of Hg. was exerted through these arteries for hours, but again without inducing rupture. It is indeed difficult to conceive of such enormous pressures existing in the living animal. Dr. Corner tells us that in his previous work in the injections of the follicular vessels of the sow he has never obtained rupture under increased arterial tension.

Conclusions

1. The theca externa of the Graafian follicle of the sow contains an abundance of typical smooth muscle cells.

2. Autonomic nerves with typical motor endings are found in juxtaposition to these muscle cells.

3. The musculature of the ovary and its follicles has a double innervation; the true sympathetics acting as inhibitory and the para-sympathetics as excitatory nerves. This innervation is similar to that of the musculature of the intestine.

4. The rupture of the follicle is not produced solely by an increase in the arterial tension of the follicular vessels.

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FIG. 1.

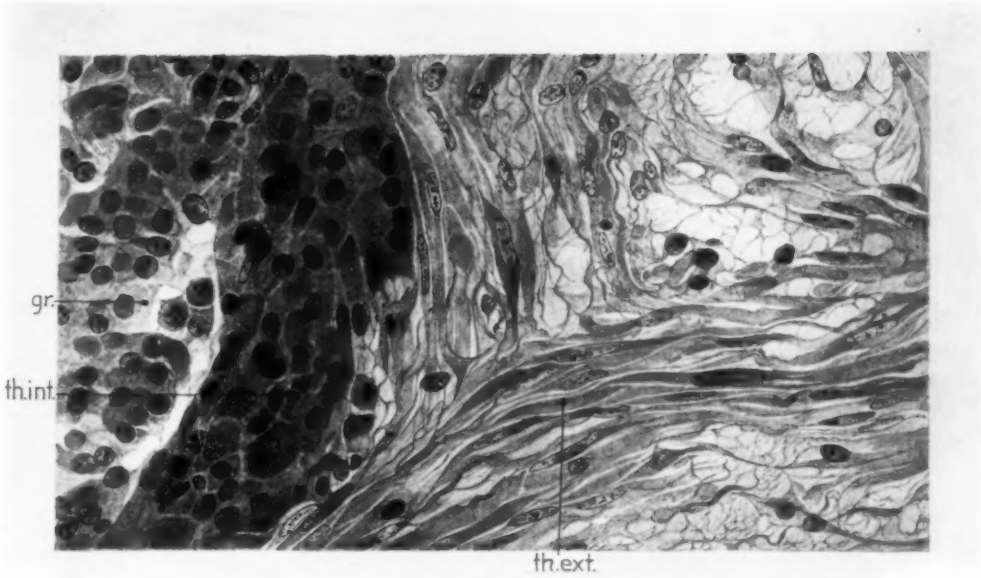

FIG. 2.

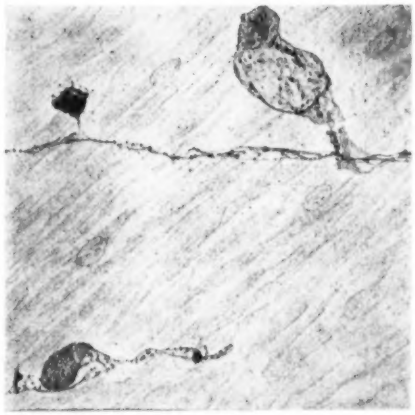

FIG. 3A.

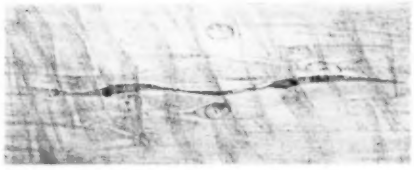

FIG. 3B.

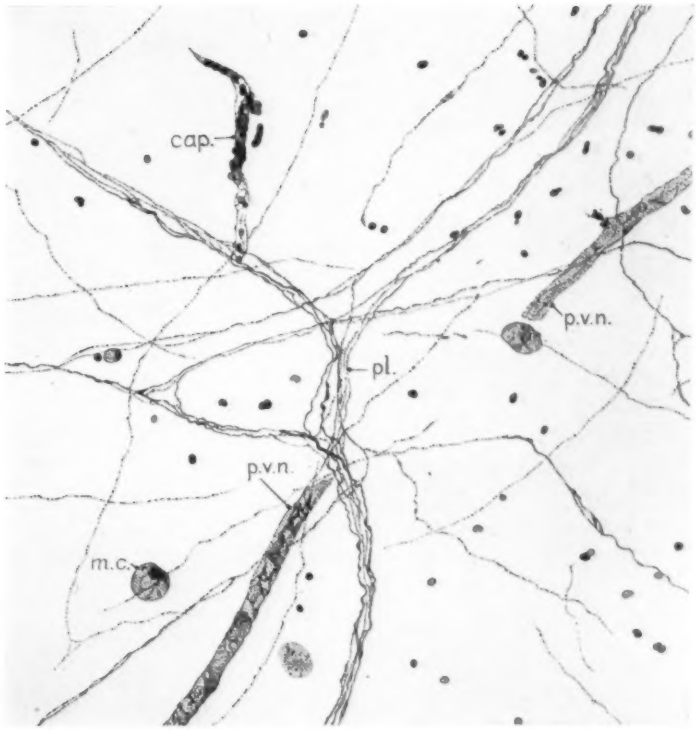

FIG. 4.

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DESCRIPTION OF PLATE

FIG. 1.—Microphotograph of the three normal layers of a mature Graafian follicle of the sow. × 90.

gr. granulosa.
th. int. theca interna.
th. ext. theca externa (the muscle-bearing layer of the follicle).

FIG. 2. Drawing of Graafian follicle immediately after rupture, showing bundles of smooth muscle cells. Van Gieson stain. × 540.

gr. granulosa.
th. int. theca interna.
th. ext. theca externa, containing numerous smooth muscle cells.

FIG. 3A.—Drawing of nerve fibrils with two types of sympathetic motor nerve endings in the theca externa of the follicle. Methylene Blue. × 975.

FIG. 3B.—Drawing of nerve fibril coursing among the muscle cells of the theca externa. Methylene Blue. × 1125.

FIG. 4.—Drawing of teased preparation of theca externa after ammonium molybdate fixation, showing rich nerve plexus and network. Methylene Blue. × 280.

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ERRATA

Attention is called to two mistakes on page 342, Plates XLIV and XLV, of the November BULLETIN in the article entitled "The Radiographic Evidence of the Influence of Cod-Liver Oil in Rickets" by Dr. E. A. Park and Dr. John Howland.

Figure 2 should be Figure 1, and Figure 1 should be Figure 2.

Figure 7 should be inverted.

JOHNS HOPKINS HOSPITAL BULLETIN

The Hospital Bulletin contains details of hospital and dispensary practice, abstracts of papers read and other proceedings of the Medical Society of the Hospital, reports of lectures, and other matters of general interest in connection with the work of the Hospital. It is issued monthly. Volume XXXII is in progress. The subscription price is $4.00 per year.

(Foreign postage, 50 cents.) Price of cloth-bound volumes, $5.00 each.

A complete index to Vols. I-XVI of the Bulletin has been issued. Price 50 cents, bound in cloth.

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The following monograph is for sale by The Johns Hopkins Press, Baltimore, Md.:

Relation of Tonsillar and Nasopharyngeal Infections to General and Systemic Disorders. By S. J. CROWE, S. SHELTON WATKINS and ALMA S. ROTHHOLTZ. 63 pages. Price, $1.75.

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